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EXPERIMENTAL PRODUCTION OF MIDGUT TUMOURS IN *PERIPLANETA AMERICANA* L.

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(*Received* 3 *October* 1957)

(With Plate 8)

INTRODUCTION

In earlier papers (Harker, 1955; 1956) it has been shown that the sub-oesophageal ganglion of *Periplaneta americana* L. undergoes a 24 hr. rhythm of secretory activity to which is related the 24 hr. rhythm of locomotor activity. Furthermore, the sub-oesophageal ganglion retains its secretory rhythm even when it is implanted into the abdomen of another animal, and the animal into which it is implanted will take up a locomotor activity rhythm in phase with the secretory rhythm of the implant, provided that the animal is not already showing a strong rhythm.

Now that this autonomous 'clock' has been found it may be possible to upset the cycle of 24 hr. to which the activity rhythm seems bound, and this might be done by continuous stimulation to activity through the ganglion secretion. Since the factors which control the secretory rhythm are change from light to darkness and the length of time spent in each of these environments within 24 hr. cycles, it is not possible to bring about continuous stimulation through the animal's own sub-oesophageal ganglion; but implanted ganglia can be used to provide a secretion during the inactive phase of the animals.

In the following pages the effect of this secondary secretion on the tissues of the alimentary canal is described. The effect on the locomotor activity rhythm will be discussed in another paper.

METHODS

Adult *P. americana* of both sexes were used in the experiments. Animals were kept in alternating light and darkness, of periods of 12 hr. each, for at least 3 weeks before the experiments were begun, and the locomotor activity rhythms were recorded to ensure that the timing of the phases of the rhythm was known. Cockroaches hereafter described as having a normal 24 hr. activity rhythm were kept in darkness from 10 p.m. to 10 a.m., those described as having a reversed rhythm were kept in darkness from 10 a.m. to 10 p.m.

Prior to the implantation of sub-oesophageal ganglia a small polythene ring was sealed with wax around a circular incision on the dorsal surface of the abdomen of each cockroach; the haemolymph flowed into the ring. The sub-oesophageal ganglia were then inserted, as rapidly as possible after dissection, and the ring was sealed with a fragment of cover-slip. It was found that the cuticle grew up the sides of the

ring rather than across the wound, so that renewal of the implant did not necessitate damaging the cuticle repeatedly. Great care was taken in making the cuticular incision to ensure that it did not penetrate the muscle layer beneath the cuticle.

EFFECT ON THE GUT OF IMPLANTED SUB-OESOPHAGEAL GANGLIA

Sub-oesophageal ganglia removed from cockroaches known to have a reversed activity rhythm were implanted, one each, into thirty-five cockroaches showing normal activity rhythms. The implants were replaced by fresh ganglia every 24 hr. at 10.0 a.m., that is at the time of change of light conditions. The implanted ganglia were sectioned after removal and it was found that they had not been encapsulated by blood cells and that the neurosecretory cells appeared to be in a normal condition.

In this series of experiments the cockroaches receiving implants were supposedly receiving secretions from the sub-oesophageal ganglia in two phases, in one phase from their own ganglion, and in the other from the implant.

The cockroaches were divided into groups of five animals, and implantation was continued for from 1 to 4 days in different groups. The treatment and the time of killing are given in Table 1. The alimentary canal of each animal was examined, and it was found that a small swelling appeared in the midgut of many. The guts of all animals were sectioned and the swellings were found to be small tumours; detailed descriptions of these are given in the next section.

Table 1. *Sub-oesophageal ganglia taken from cockroaches with reversed 24 hr. locomotor activity rhythms and implanted into cockroaches with normal rhythms.*

Groups of five animals	Days									
	1	2	3	4	6	8	10	12	14	16
A	Implanted	Implanted	Implanted	Implanted	—	Killed, no tumour	—	—	—	—
B	Implanted	Implanted	Implanted	Implanted	—	—	Killed, small tumour in two animals	—	—	—
C	Implanted	Implanted	Implanted	Implanted	—	—	—	Killed, small tumour in two animals	—	—
D	Implanted	Implanted	Implanted	Implanted	—	—	—	—	Killed, tumour in all animals	—
E	Implanted	Implanted	Implanted	Implanted	—	—	—	—	—	Killed, tumour all anim
F	Implanted	Implanted	—	—	—	—	—	—	—	Killed, tumour in three animals
G	Implanted	—	—	—	—	—	—	—	—	Killed, n tumour

It can be seen from Table 1 that when implantation was performed from two to four times the recipient cockroaches, in all but one case, formed tumours by the sixteenth day from the beginning of the experiment. The tumours all appeared in the midgut, irrespective of the region of the abdomen in which the implant was made. To confirm the lack of dependence of the position of the tumour on the

position of the implant, implantation into the thorax was performed daily for 4 days on three cockroaches; at the end of 18 days a tumour was found in the midgut of each animal.

As a control similar implantations were made into two groups of five animals each, but, instead of sub-oesophageal ganglia, brains were implanted in one group and corpora allata in the other. Implantation was repeated for 8 days in each case. There was no sign of tumour formation, after 28 days, in any of the animals.

Sub-oesophageal ganglia taken from cockroaches showing normal activity rhythms were implanted into ten cockroaches which also had normal activity rhythms. In this series of experiments the recipient cockroaches were supposedly receiving secretions from both sub-oesophageal ganglia at the same time, that is, during only part of the 24 hr. Implantation was continued daily for 4 days in five animals, and for 8 days in five animals. None of the animals showed any sign of tumour formation, even after 28 days from the beginning of the experiment.

Extracts of sub-oesophageal ganglia were prepared by grinding a single ganglion in 0·05 ml. of Ringer solution, and a fresh extract was injected into the abdomen of each of five cockroaches during the inactive phase. This was repeated daily for 12 days. No tumours were formed in any of these animals. Similar extracts were prepared using five ganglia in 0·05 ml. of Ringer solution, and these were injected daily for 5 days into five other animals. After 21 days three of the cockroaches were found to have a small tumour in the midgut. An extract of this strength (five ganglia in 0·05 ml. Ringer) injected into five cockroaches every day during their active phase, and repeated for 5 days, did not produce tumours in any of the animals.

Previous experiments (Harker, 1956 and unpublished) have shown that, although phases of the locomotor activity rhythm of *Periplaneta* can be determined by the phases of the light: dark rhythm to which they are subjected, not all the processes in the animal which undergo a 24 hr. rhythm are controlled in this way. For instance, there is a variation over 24 hr. in the type of reaction of headless cockroaches to injections of sub-oesophageal ganglia extracts, and the type of reaction appears to be related to the time of day and not to the light: dark treatment, nor to the resultant locomotor activity rhythm of the animal before decapitation. It seems likely then that, although the phases of the locomotor activity rhythm are reversed in cockroaches which have been subjected to reversed light and darkness, the phases of some metabolic rhythms persist in the normal form. If this is so, cockroaches might show some variation at different times of day in their sensitivity to implanted sub-oesophageal ganglia; the following experiment was performed to test this hypothesis.

Five cockroaches were kept in reversed light and darkness for 3 weeks; their locomotor activity rhythms were found to be reversed. Into these cockroaches were implanted sub-oesophageal ganglia taken from animals with normal activity rhythms; implantation was repeated daily for 4 days. No tumours had formed in these animals by the twentieth day. The experiments were repeated, this time the treatment was continued for 8 days. Two of the animals had formed tumours in the midgut by the twentieth day. In this experiment the secretory activity of the

sub-oesophageal ganglia is supposedly similar to that in the previous experiments, yet the incidence of tumours is lower and treatment had to be continued for a longer period before tumours were formed. It seems likely that either the gut shows a rhythm of sensitivity to sub-oesophageal secretion, a rhythm which is not reversed by reversed light:dark conditions, or that some other organ affected by the sub-oesophageal ganglia secretion and involved in tumour production has a rhythm independent of light:dark conditions. In either case it appears that *Periplaneta* is not as sensitive to the sub-oesophageal ganglion hormone at the time when the hormone would normally be present as at the time when normally none would be present.

HISTOLOGY AND THE DEVELOPMENT OF THE TUMOURS

The histology of the normal midgut of *P. americana* has been described by Day & Powning (1949). There is an outer layer of scattered strands of longitudinal muscle, inside of which run the circular muscles. Between the inner epithelium and the circular muscles is a distinct region of connective tissue. The epithelial cells are separated into groups, more clearly so at the anterior end of the midgut, by the presence of nidi of regenerative cells; the cells of the nidi replace degenerating epithelial cells, and these degenerating cells are always those furthest from the nidi (Pl. 8 (i)). In the normal midgut the nidi are evenly distributed and the number of epithelial cells between nidi is practically constant. The number of cells in each nidus in well-fed animals is also fairly constant. Counts were made of these cells and of the number of mitoses per twenty-five nidi in the normal midgut; the results appear in Table 2.

Table 2

	Normal midgut	Midgut of treated animals		
		2 days after implantation	4 days after implantation	8 days after implantation
Mitoses per 25 nidi, means of 10 counts	8·1	18·0	18·3	Nidi not clearly defined
Cells per nidus, means of 50 counts	8 (8–10)	16 (16–18)	16 (16–18)	Nidi not clearly defined
No. epithelial cells between nidi, means of 50 counts	16	28	30	30

Histological examination was made of the midguts of cockroaches fixed at 24 hr. intervals from the beginning of experiments in which sub-oesophageal ganglia from insects with reversed rhythms were being implanted into insects with normal rhythms. From 2 days after the beginning of the experiments areas of tissue could be seen in which the number of mitoses had increased and in which the clear pattern of cells had become slightly distorted (Pl. 8 (ii–iv)). The following descriptions apply only to these regions. Counts were made of the number of mitoses per twenty-five nidi, the number of cells in each nidus, and the number of epithelial cells between neighbouring nidi. It is difficult to delimit with any certainty the region

affected, and the numbers given in Table 2 cannot be taken as accurate; where they err it will be because normal cells have been included and so the numbers will be low.

From the results appearing in Table 2 it can be seen that the number of mitoses increases rapidly after treatment, more than doubling after 2 days; the number of cells in each nidus also increases, but it appears that some cells are moving up into the epithelium. Seven days after the beginning of treatment, in the majority of cases, there is a double line of cells in the epithelium (Pl. 8 (ii), (iv)); in determining this, it is necessary to ensure that sections are cut at right angles to the main axis of the gut, and that the region of suspected tumour formation is not at either end of the midgut, where a double line of epithelial cells may normally be present.

At the same time as the mitotic activity increases, that is from the second day, cells from the nidi are seen to have moved into the connective tissue layer adjacent to the muscle layer; it appears to be these cells which form the tumour. After 14–18 days the epithelial cells begin to break down (Pl. 8 (iv), (v)); the tumour then consists of small cells with very little cytoplasm and which stain densely with haematoxylin; these cells form a whorled pattern and a number of tracheae run between them.

Small metastases have been found in eight cockroaches after 24–30 days; they appear in other regions of the midgut, the foregut and hindgut and in the salivary glands (Pl. 8 (vii), (viii)). The metastases may arise from cells moving in the connective tissue layer of the gut as well as in the haemocoele, for cells appear to migrate in both.

Transplants of small portions of the tumours, about 0·5–1·0 mm.^{2}, were placed in the haemocoele of twenty cockroaches. The implants from two animals were examined after 2 days and were seen to be surrounded by a sheath of blood cells. Two cockroaches were killed every 2 days for 14 days, and it appeared that the transplanted portions had increased in size; however, after 4 days it is difficult to differentiate between the surrounding blood cells and the transplanted cells. Of the remaining cockroaches, two were examined at 20, 24 and 28 days respectively; three of these six animals had developed tumours in the midgut, and one in the hindgut.

Small portions of normal gut tissue approximately equal in size to the transplants of tumour tissue were implanted into ten control animals. None of these animals showed any sign of tumour formation after 28 days.

The sequence of tumour formation was examined in three groups of four animals into which tumour tissue had been transplanted 8, 14 and 28 days previously. The sequence appeared to differ in some details from that of primary tumour formation. Eight days after the transplantation had been made cells were seen to be invading the connective tissue layer below the nidi of the midgut; the width of the connective tissue layer increased about fourfold in the next 5 days (Pl. 8 (ix)), and it was not until this stage was reached that the epithelial cells (in all but one animal) showed any abnormal appearance. In the one exception the epithelial cells had begun to degenerate by the eighth day. The nidi had lost their normal form by the eighth day in two animals, and in all four of the animals killed on the fourteenth day. The nidi of the surrounding tissue showed no increase in mitosis until after the epithelium

had begun to degenerate; in this type of tumour formation it appears that the nidi do not supply cells to the tumour, but only carry out their normal function of replacing epithelial cells. After the epithelium had begun to break down, that is, between the eighth and fourteenth day, blood cells began to accumulate along the muscle layer and these added superficially to the size of the swelling. The swelling at this stage is invaded by tracheae.

In insects swellings due to wound healing have something of the appearance of malignant growth (Day, 1952; Wigglesworth, 1937), but that neither the primary nor transplanted tumours are swellings of this type appears evident (i) from the control experiments, (ii) from the fact that in the formation of primary tumours the site of implantation is not related to the region in which the tumour is formed, and (iii) from the evidence of increase in mitotic activity of the cells of the nidi.

TEMPORARY CONTROL OF THE TUMOURS

Ten groups of three cockroaches were implanted daily for 4 days. Sub-oesophageal ganglia taken from cockroaches with reversed activity rhythms were implanted daily for 4 days into ten groups of three cockroaches each. Implantation was discontinued from day 4 to day 10. On day 10 one group was killed; tumours were found to be developing in all three animals, and had reached the stage in which the number of epithelial cells had increased, but there was no breakdown as yet of the epithelial tissue (see Table 3). Daily implantations were made into the remaining groups from day 10 until day 20, using sub-oesophageal ganglia from animals whose rhythms were normal and in phase with those of the recipient cockroaches. Every second day one group of animals was killed and the guts sectioned. The sections showed that tumours had not developed beyond the stage already reached when the second series of implantations began on day 10. The number of mitoses in the nidi had apparently returned to normal, but the affected region was recognizable by the increased number of cells. On day 20 implantation was discontinued and the examination of one group every 2 days continued. By day 24 the tumour size had increased considerably, the epithelial layer had been broken down, and the general appearance was that of a mass of small cells in whorled formation. This stage is referred to as 'stage 14 days' in Table 3; the term is only one of convenience and it is not claimed that certain stages are closely correlated with the number of days from the beginning of the treatment.

In this experiment the presence of excess sub-oesophageal ganglion hormone at the time when some secretion would normally be present appears to control the growth of the tumours. That the cells of the tumour have undergone an irreversible change, however, is shown by the increased growth as soon as the hormone level returns to normal.

The number of animals used in this experiment, as in those previously described, has had to be limited because of the time which every operation takes, and because of the large numbers of cockroaches necessary to supply the sub-oesophageal ganglia for implantation.

Table 3. *Sub-oesophageal ganglia taken from cockroaches with reversed activity rhythms, R; taken from cockroaches with normal activity rhythms, N. Recipient cockroaches all had normal activity rhythms*

Groups of three animals	Days											
	1–4	4–10	10	12	14	16	18	20	22	24	26	28
A	Implanted, R	—	Killed, tumours in all at 'stage 10 days'*	—	—	—	—	—	—	—	—	—
B	Implanted, R	—	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—	—	—	—	—
C	Implanted, R	—	Implanted, N	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—	—	—	—
D	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—	—	—
E	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—	—
F	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—
G	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	—	Killed, tumours at 'stage 12 days'	—	—	—
H	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	—	—	Killed, tumours at 'stage 14 days'	—	—
I	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	—	—	—	Killed, tumours at 'stage 16 days'	—
J	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	—	—	—	—	Killed, tumours large

* For explanation of term 'stage *x* days' see text.

DISCUSSION

The three main conclusions which might be drawn from these results are that the presence of excess amounts of sub-oesophageal ganglion secretion brings about formation of transplantable tumours in the gut of *Periplaneta*, that the midgut is the region most frequently affected, and lastly, the most striking conclusion, that the *timing* of the presence of the secretion is of first importance in tumour formation.

The importance of timing, both in the production of tumourous cells and in the growth of the tumours, apparently results from the interaction of 24 hr. rhythms of several processes. Although the introduction at any time of day of large excesses of hormone is followed by the formation of tumourous cells, at a low level of concentration not only is the 24 hr. rhythm of secretion concerned, but also the sensitivity of the gut, which itself shows a 24 hr. rhythm. The hormone continues to act when the cells have become tumourous and again it is not just the presence of the hormone which is involved, but the timing of its presence, for this same hormone which was responsible for the formation of the tumour can control its growth, if present at the normal time. Perhaps it is also significant that the cells which give rise to the tumour are cells which probably show a 24 hr. rhythm of mitosis under normal conditions; this has not been shown, but it appears likely, since the regeneration of the epithelium is connected with feeding, and feeding follows the 24 hr. rhythm of locomotory activity.

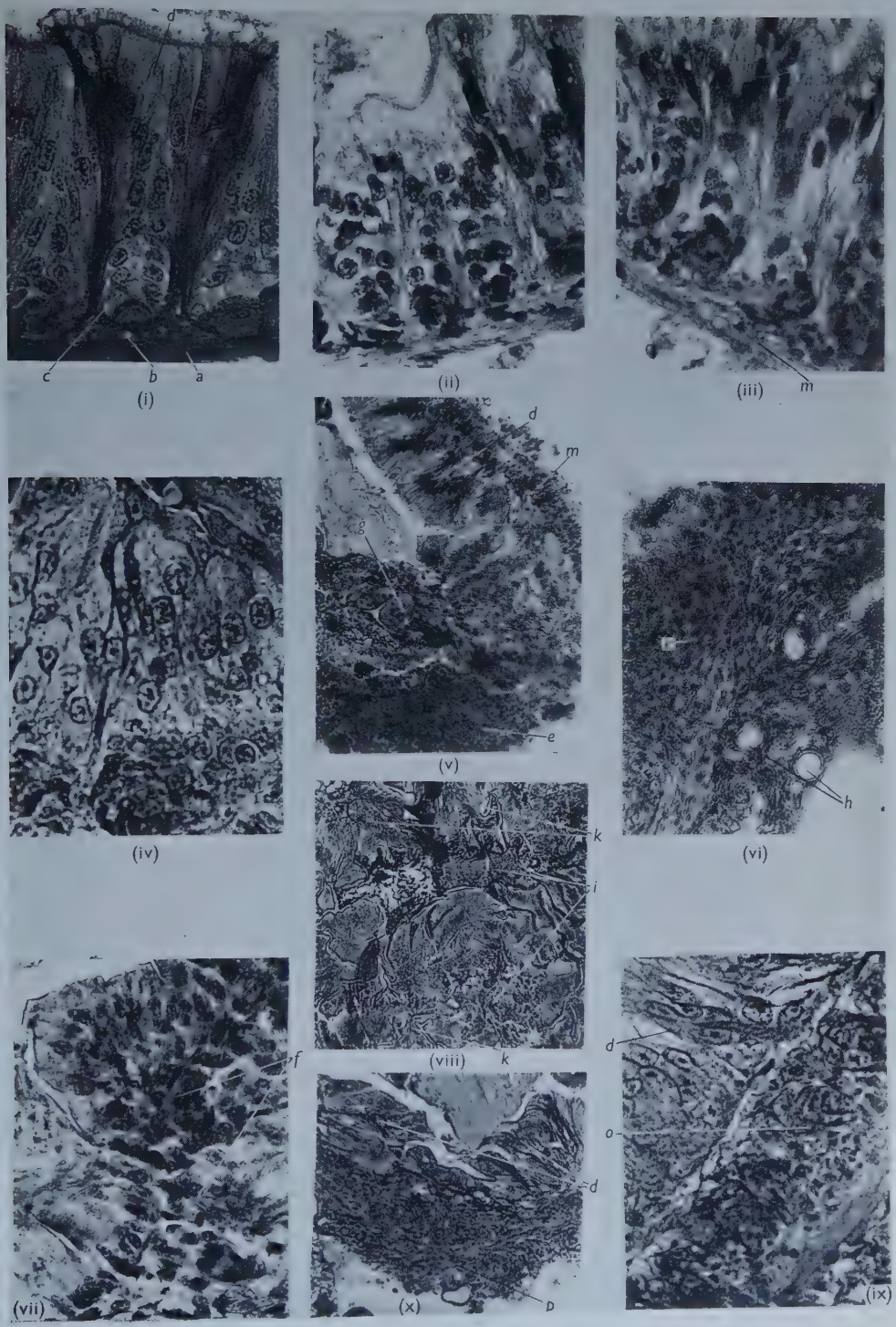
Nothing is known of the chemical nature of the sub-oesophageal ganglion secretion, nor of the way in which it acts on the animal in regulating the locomotor activity rhythm. Whether it acts through one or several systems, and whether it is the secretion itself or a secondary system which is responsible for the formation of tumours, is unknown, but, however tumour formation is brought about, the essential feature appears to be the continuous presence of the hormone. The normal animal seems to be well safeguarded from continuous secretion; even if the sub-oesophageal ganglion could maintain a continuous supply, the secretory phase appears to be bound to a 24 hr. rhythm which has, as yet, proved unalterable by experimental means.

SUMMARY

1. Sub-oesophageal ganglia taken from *Periplaneta americana* L. which had been kept in reversed conditions of light and darkness have been implanted into the abdomens of cockroaches living in normal light : dark conditions. When implantation was continued for 4 days transplantable tumours appeared by the eighteenth day in the midgut of the majority of cockroaches.

2. No tumours appeared in the guts of cockroaches when the implanted sub-oesophageal ganglia had been taken from animals kept in normal conditions of light and darkness, nor when brains or corpora allata were implanted.

3. Injection of an extract of five sub-oesophageal ganglia in 0·05 ml. Ringer solution produced tumours only when injection took place during the inactive phase of the 24 hr. locomotory rhythm.



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(*Facing p.* 258)

4. The implantation of sub-oesophageal ganglia taken from animals kept in normal light:dark conditions into animals kept in reversed light:dark conditions was followed by tumour formation only if the implantation was carried out daily for 8 days. The incidence of tumour formation is lower than under the conditions described in 1.

5. Details are given of the histology of the developing tumours.

6. When tumour formation had begun the implantation of sub-oesophageal ganglia with secretory phases in phase with that of the animal's own ganglion prevented further growth of the tumour. The cells of the tumour had, however, undergone an irreversible change for, as soon as implantation ceased, growth began once more.

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EXPLANATION OF PLATE 8

(i) T.S. normal midgut of *Periplaneta americana*. *a*, circular muscle; *b*, connective tissue; *c*, nidus of regenerative cells; *d*, epithelial cells; × 500.

(ii) T.S. midgut. Nidus cells and epithelial cells have begun to multiply; × 430.

(iii) T.S. midgut. Further increase in epithelial cells, beginning of breakdown of nidi, movement of cells from nidi into connective tissue (*m*); × 430.

(iv) T.S. midgut. Break down of clear pattern of epithelial cells; × 430.

(v) T.S. midgut. Small cells of tumour (*e*), normal epithelium (*d*), cells invading connective tissue (*m*), broken down epithelium (*g*); × 100.

(vi) T.S. tumour showing small densely staining cells (*e*) and invading tracheae (*h*); × 430.

(vii) Early metastasis in hindgut (*f*); × 430.

(viii) Early metastasis in foregut (*i*), normal cells (*k*); × 100.

(ix) T.S. midgut. Invasion of connective tissue by cells (*o*) from transplanted tumour; normal epithelium (*d*); × 430.

(x) T.S. midgut. Small metastasis (*p*) from transplanted tumour; the epithelial cells (*d*) have not been broken down; × 100.

THE BLOOD VOLUMES OF SOME REPRESENTATIVE MOLLUSCS*

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(*Received* 10 *September* 1957)

The reports of measurements of the blood volume of molluscs found in the literature are few in number and represent, in most cases, incidental observations rather than results of specific investigations. Leitch (1916) obtained by the bleeding of *Planorbis* a volume of blood which was 33% of the tissue weight. In 1931, Borden determined the blood volume of *Planorbis corneus* using the Welcker method and obtained a value of 58% of the total tissue weight. This higher value is to be expected, since it is impossible to remove all the blood from an animal by simple bleeding. In 1950, Martin & Huston, using the inulin dilution method, reported the blood volume of *Aplysia californicus* to be 83·3% of the total weight. This is not surprising since *Aplysia* has a very extensive haemocoele, and more than half the body weight in blood can be drained in a few minutes from a single incision into the animal.

Prosser & Weinstein (1950) determined the blood volume of two fresh-water molluscs, *Lampsilis ventricosa* and *Amblema costata*, and found a very low value. In terms of percentage of total wet tissue weight, their average values for experiments done with sodium thiocyanate and with T-1824 are very close to 8%. These results are not verified by the brief report of Potts (1954) who, in studying the rate of urine production in *Anodonta cygnea*, found a mean inulin space of four animals to be 55% of the wet tissue weight.

Since the values which have been reported are so few in number and are so wide in range a more extensive study of the subject appeared to be of value. The purpose of this investigation was to measure the total volume of circulating fluid in members of various groups of molluscs. From the results obtained the extent of variability within certain species and, to some extent, within the phylum can be ascertained.

GENERAL METHODS

Blood volumes were determined by measuring the dilution of a known quantity of material injected into the circulating fluid. In order to facilitate rapid mixing of the

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‡ Contribution No. 98 of the Marine Biological Laboratory.

material, it is desirable to make injections directly into the heart or a major blood vessel. When it was impossible to use this technique, as was the case in about half the forms investigated, the injections were made directly into the haemocoele.

Table 1. *Summary of experimental procedures*

Animal	Wet wt. without shell (g.)	Vol. of initial normal blood sample (ml.)	Material injected	Vol. injected (ml.)	Vol. of each serial sample (ml.)	Sampling interval (min.)	Total length of experiment (min.)
yptochiton stelleri	421–877	5–7	Inulin, 0·4% in blood	5	0·5	30–60	300
lysia californicus	146–411	2–5	Rabbit Hb 3% in blood	7–20	2	15–30	60–235
			Inulin, 0·5% in blood	6–10	1–2	15–30	180–400
			0·05–0·10% silver proteinate in blood	8–15	1–2	15–30	85–400
chidoris sp.	50–62	0·5–1·0	Inulin, 0·5% in sea water	1–2	0·2–0·5	20–30	240–360
hatina fulica	41–74	1·0	Inulin, 0·5–1% in distilled water	1	0·2	15–20	130–270
ion ater	10·0–43·7	0·15	Inulin, 4% in distilled water	0·20	0·05	10–60	120–480
ytilus californianus	39–122	1–1·5	Inulin, 1% in sea water	0·5–1·0	0·2	15–30	180–250
argaritana margaritifera	31–49	0·5–1·0	Inulin, 1% in distilled water	0·5–1·0	0·15–0·20	15	120–180
			T-1824 bound to rabbit serum	0·5–1·0	0·15–0·20	15	120–180
topus hongkongensis	4550–22500	7–10	T-1824, 0·1–0·375 in sea water	3–15	1·0–2	15–30	135–540
			HgS, 2·3% in sea water	10	1·0	30	450–495
			Inulin, 4–5% in sea water	8–100	1·0–2	15–30	360–540

A number of agents were tested as to their applicability in blood volume determinations. These included various dyes, haemoglobin, haemocyanin, mild silver proteinate, sucrose, and inulin. The most satisfactory material used was inulin as it did not appear to complex with any portion of the circulating fluid and was only slowly excreted. The material to be injected was prepared by diluting a stock aqueous solution with blood from animals of the same species or with a fluid of approximately the same osmotic concentration as the circulating fluid. At the beginning of the experiment enough normal blood was withdrawn for preparation of blanks and standards. The volume of test solution injected approximately replaced the amount of blood removed for this purpose. Samples of blood were taken at appropriate intervals over a period of time which preliminary experiments had shown to be sufficient for uniform distribution. Before taking each sample a quantity of blood larger than the dead space of the sampling tube was removed and subsequently re-injected. In Table 1 are summarized the specific details concerning the experimental procedure followed for each form investigated.

Standard analytical procedures were used in the determination of the concentra-

tion of the materials employed. For those analyses which required a protein-free filtrate, the proteins were precipitated by the Somogyi method (1930). The concentration of various dyes and other coloured materials were determined by comparison of the optical density of the experimental samples to those of standard solutions of known concentration which were prepared simultaneously. Inulin was analysed by the Harrison method (1942) and the anthrone method (Morris, 1948). For the determination of inulin in samples from animals with a relatively high blood sugar, a yeast digestion was employed with the Harrison method and a NaOH digestion (Little, 1949) with the anthrone method to remove the interfering sugar. The instruments used in different laboratories included the Beckman DU spectrophotometer, the Klett–Summerson photoelectric colorimeter and the Cenco Photelometer.

For the calculation of blood volume the general procedure was to plot the optical density of the samples against time. Since the concentration of the injected material decreases exponentially with time, semi-logarithmic graph paper was used to obtain a linear relationship. The line obtained was extrapolated back to zero and the concentration in grams per millilitre equivalent to the optical density at zero time was determined from a standard curve. The volume of fluid into which the injection had been made originally was obtained by dividing the weight of the injected material by the concentration at zero time. This volume of fluid was then expressed as percentage of the wet weight of the animal without shell.

Blood volumes, cell water volumes and total water volumes are expressed as 'mean ± standard deviation %' of the wet weight of the animal or tissue.

ANIMALS AND SPECIAL METHODS

A. *Placophora*

The volume of circulating fluid was determined in the large chiton, *Cryptochiton stelleri*. The animals which occur in abundance along the shores of Puget Sound were collected at approximately 3-month intervals. They were maintained at 8–10° C. in 150 gal. aquaria through which filtered sea water was recirculated at a rate of approximately 1 l. per minute per aquarium. A description of the sea-water system in which these animals were kept may be found in Thompson (1935). Under these conditions the animals survived in healthy condition for as long as 2 years.

In preparation for an experiment each animal was removed from the sea water, blotted dry and weighed. In this form, blood samples were taken through a plastic catheter placed in the haemocoele. For insertion of the catheter, a needle of smaller diameter was forced through the body wall in a region below the middle shell plates. The plastic catheter stiffened with an internal wire was then pushed through the opening, the internal wire was withdrawn and the tube plugged to prevent bleeding. This technique resulted in a leak-proof opening into the haemocoele through which fluid could be injected or withdrawn. Blood samples were often expressed from the catheter under pressure, not from action of the heart but from contraction of the body wall. Occasionally blood contaminated with eggs was obtained; such an animal was omitted from the report. Many specimens were dried to constant weight at 105° C. and the total body water calculated after correcting for the shell weight.

B. *Gastropoda*

(1) *Opisthobranchia–Tectibranchiata*

The sea hare, *Aplysia californicus*, was collected at low tide at about 2-week intervals on the Southern California coast in the vicinity of Corona del Mar. They were maintained on a diet of algae and kept in a running sea-water aquarium until needed.

At the beginning of the experiments the animals were wiped dry, weighed, and then a multiply perforated plastic catheter was inserted through an opening made in the body wall. The tube was tied in place with a ligature which caught up a small portion of the tissue. Since this animal is able to contract the body so as to close off a surprising length of tubing it was not until catheters of half body length were inserted that successful sampling became routine. Since there was a loss in weight due to the release of fluid from glands in the handling procedure and from the escape of blood during cannulation of the animal, another weight determination was made before the injection of the experimental solution. The weight determined at this time, corrected for the contents of the gut, was used subsequently in calculation of the blood volume. The injections were made into the ctenidial vessel, and samples were taken from the catheter in the body wall.

At the end of the experimental period the body wall was cut and the volume of blood which could be drained from the animal was determined. The contents of the digestive tract were removed and weighed and the animals dried to constant weight at 105° C.

(2) *Opisthobranchia–Nudibranchiata*

The sea lemon, *Archidoris* sp., was the animal used as a representative of the nudibranch molluscs. Specimens were collected intertidally along the shores of San Juan Island and were maintained in the all-glass sea-water system of the Friday Harbor laboratories.

The technique used for cannulation was the same as that described for *Aplysia*. However, in this form injections were made and samples subsequently withdrawn from the catheter in the haemocoele. Consequently, before the experimental samples were taken, the blood of the catheter was thoroughly mixed with the blood of the haemocoele by flushing it in and out several times. The amazingly effective reflexes of the animal sometimes resulted in contractions of the body wall which succeeded in shutting off all the apertures in the cannula. However, the animal would relax eventually and samples could then be taken successfully. At the end of the experiment the animals were bled and dried to constant weight in an oven at 105° C.

(3) *Pulmonata*

The giant African snail, *Achatina fulica*, was collected in the vicinity of Kaneohe Bay on the island of Oahu, Territory of Hawaii. The animals were kept in glass terraria provided with a sod bottom and given water and fruit *ad lib.*

The animals used in these determinations were anaesthetized with ether vapour in a closed container, the process taking about ½ hr. When the animals were sufficiently relaxed two stitches with linen thread were taken close together through the body wall slightly posterior to the genital aperture. Between the stitches a small opening was made and the soft plastic cannula inserted through the opening into the haemocoele. The cannula was tied securely in place to prevent leakage of blood. In about 30 min. the animals appeared to have recovered from the anaesthesia and began to crawl about in the customary fashion. At this time the injection was made into the haemocoele, and then at subsequent intervals of time blood samples were removed from the same cannula. At the end of an experiment

the shell, and any eggs that might be present were removed, the free blood was drained off, and the remainder of the tissue dried to constant weight.

The slug, *Arion ater*, was collected for the most part on the campus of the University of Washington, in Seattle, and at Friday Harbor. The individuals were maintained in terraria with an ample supply of water, lettuce and oatmeal.

Collection of blood and injection of materials were made through a plastic catheter which was inserted through the lateral body wall in the middle region of the animal. In this form it was not necessary to tie the catheter in place. If the animals were cooled to about 8° C. before the insertion of the catheter, the production of mucus and general body movements were decreased, making the operation easier.

In the early experiments, the dry weights of the animals were not determined. However, they were obtained for later experiments by the drying of the specimens to constant weight in an oven at 105° C.

C. *Pelecypoda*

Filibranchiata

The mussel, *Mytilus californianus*, was collected intertidally along the breakwater south of the entrance to Gray's Harbor, Washington. Specimens were kept in the Seattle sea-water system described earlier.

Preparation for the experiments was made without anaesthesia. After scraping away part of the hinge ligament, always working between known positions of the muscles, it was possible to break away small pieces of shell with a surgical rongeur or bone forceps. The larger part of the heart and most of the aorta were exposed by this method. It was necessary to remove the shell carefully to prevent damage to the body wall or muscles. The animal was weighed and then placed in a container of sea water of such depth as to leave only the exposed area free for injection and sampling. Operated animals which were not used for experimentation survived well in the laboratory and frequently would begin to deposit shell.

After such preparation a sharp 1 in. hypodermic needle of 26 or 27 gauge was introduced directly into the anterior aorta, and anchored in place with a piece of modelling clay resting on the shell of the animal. The test solution, coloured with T-1824, was injected into the aorta and the preparation carefully examined for leaks. During the course of the experiment, blood samples were withdrawn at appropriate intervals of time. At the completion of the experiment the animal was weighed and the volume of blood drained from multiple cuts in the region of the heart determined. The animal was removed from the shell and the weight of the shell recorded as well as the wet weight of the tissue. The dry weight of the tissue was determined after drying at 105° C.

Specimens of *Margaritana margaritifera* were obtained from the shallow waters of the Sammamish river in King County, Washington, about halfway between the origin of the river in Lake Sammamish and its termination in Lake Washington. They were kept in about 4 in. of water in a cement aquarium table through which Seattle tap water circulated at a slow rate. This water is chlorinated from time to time and does not contain much plankton. Experimental animals were not kept longer than a month before use, though some left in this aquarium survived for several months.

In preparation for an experiment a triangular section of a valve in the area of the heart and between the attachments of the adductor muscles was prised out after circumscribing the area with hacksaw cuts. The opening was extended if necessary by chipping away additional pieces of the shell. The animal was weighed and returned to a container through which fresh tap water circulated.

Since it is difficult to see the aorta in this form, a sharp 26 or 27 gauge hypodermic needle

was thrust through the pericardial sac into the lumen of the ventricle and supported firmly by a clamp. Into the end of the needle was inserted an adapter of small internal diameter to which was connected a 3 in. length of plastic tubing (1 mm. I.D.). Injections were made and samples were taken from the end of the plastic tubing. The use of this sampling device was advantageous, since it had a very small dead space and eliminated any movements during sampling which might destroy the fragile preparation. At the close of the sampling period weight and volume determinations were made similar to those for *Mytilus*.

D. *Cephalopoda*

Specimens of the large octopus in Puget Sound, probably *Octopus hongkongensis* Hoyle (Pickford, personal communication), were taken from their dens at low tide or captured on the rocks in shallow water. They were kept in large aquaria either at the Friday Harbor Laboratories of the University of Washington, or in the sea-water system at Seattle which has already been described. In most instances the animals ate the crabs or fish offered to them and could be kept indefinitely. In a few cases the animals would not accept food during the week or two which preceded their use in the experimental work.

Surgical provision was made for obtaining blood samples, usually the evening before the determination. The octopus was anaesthetized in about 30 gal. of aerated sea water by the gradual addition of 95% ethyl alcohol to a final concentration of about 2·5%. In 15–30 min. relaxation of the animal was obtained, at which time one of the branchial hearts could be drawn out of the mantle cavity without damage to the animal. An opening was made through the wall of the branchial heart and a soft rubber catheter passed through the lumen of the heart and into the lateral vena cava. It was secured with a ligature which enclosed a complete ring of tissue. The external end of the tube was closed with a solid glass stopper, the animal weighed and returned to normal sea water. In a short time the normal respiratory movements were regained, as well as muscular control. To prevent the animal from disturbing the tube, it became the practice to tie the tentacles in a net bag immediately after recovery from anaesthesia.

The next day, in preparation for an experiment, a small bore plastic tube long enough to hang over the edge of the aquarium was connected to the rubber catheter and the net bag was removed from the animal. Solutions were injected and blood samples were taken through the same tube which was adequately rinsed both for complete injection and to insure the taking of representative blood samples.

RESULTS

A. *Placophora*

Early experiments on *Cryptochiton* showed that a linear relationship between time and the logarithm of the concentration of the injected material was not obtained until 1–2 hr. had elapsed. The period of time necessary for this evidence of uniform distribution will be referred to subsequently as the equilibration time. In order not to remove too much of the injected materials before mixing had occurred, the first sample was taken after approximately an hour had elapsed. In Fig. 1 are given data from a typical experiment on the species. The long periods required for distribution of the injected material probably indicate a sluggish circulation in this animal. Although samples were taken for a long period, the possibility exists that not all the injection fluid entered regions through which free circulation occurred. It is thought that this might account for some of the variability in the results obtained. Since the

weight of the valves which make up the shell, and the total body water, were not determined for each experimental animal, these quantities were determined for a series of five normal animals. The valves averaged 5·2% of the total wet weight, and the total body water averaged 85·1 ± 2·2% of the wet tissue weight.

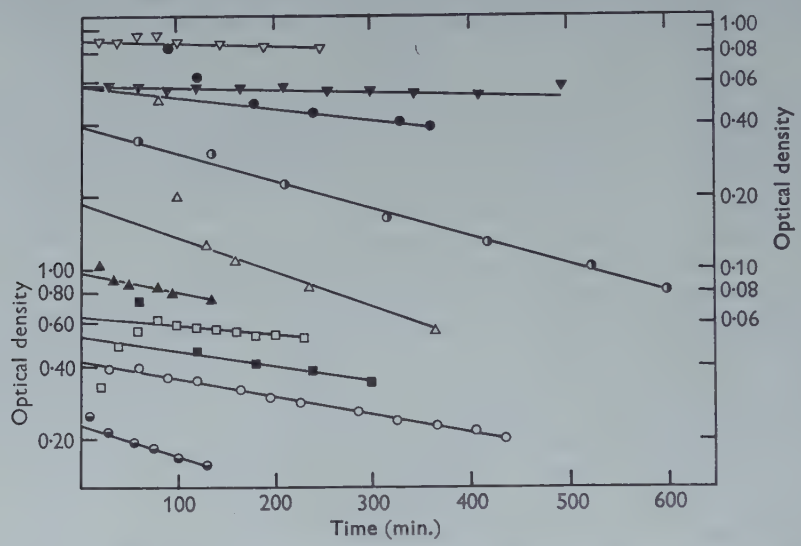

Fig. 1. Typical dilution curves following the injection of inulin into the circulatory system of the following animals: Group A. Use scale on right ordinate. Group B. Use scale on left ordinate. A: ▽, *Aplysia californicus;* ▼, *Octopus hongkongensis* (HgS); ●, *Octopus hongkongensis* (inulin); ◑, *Arion ater;* △, *Archidoris* sp. B: ▲, *Achatina fulica;* □, *Mytilus californianus;* ■, *Cryptochiton stelleri;* ○, *Octopus hongkongensis* (T-1824); ◒, *Margaritana margaritifera.*

In Table 2 the blood volumes of fifteen animals are given. The average blood volume was 43·8 ± 8·6%. Also included in this table are results of blood-protein determinations and values for cellular water. The protein was analysed by a micro-Kjeldahl method (Ballentine & Gregg, 1947). The percentage cellular water was calculated in the following manner. Since there is an open circulatory system in *Cryptochiton*, the volume of cellular water was obtained by subtracting the blood water from the total volume of water in the animal. To the volume of cellular water was added the dry weight of the animal, corrected for the shell and the salt and protein content of the blood, if this dry weight was significantly large. This sum theoretically should be equivalent to the wet weight of the cells in the tissues. The volume of cellular water was then expressed as percentage of the wet weight of the cells. The cellular water for this animal was 76·6 ± 3·4%. A discussion of the cellular water of this and the other forms studied will be deferred until later in the paper.

Table 2. *Inulin volume, blood protein and cellular water of* Cryptochiton stelleri

Wet wt. without shell (g.)	Blood vol. (ml.)	Blood vol. as % wet wt. without shell	Blood* water (ml.)	Blood protein g./100 ml.	Dry wt. without shell (g.)	Corrected† dry wt. (g.)	Cellular water as % wet tissue wt.
421	250	59·4	246	0·97	62·6	52·7	68·1
475	230	48·5	224	2·47	70·8	58·2	75·5
484	266	54·9	259	—	72·1	58·8	72·2
495	204	41·2	199	—	73·8	63·6	77·8
578	185	32·0	180	2·55	86·2	75·9	80·4
649	292	45·0	283	2·94	96·6	79·3	77·3
666	367	55·1	354	3·58	99·3	75·2	74·0
670	222	33·1	217	—	99·7	88·6	79·9
712	308	43·2	300	—	106·0	90·6	77·1
726	381	52·5	372	—	108·2	89·2	73·3
753	273	36·3	266	2·25	112·2	97·8	79·3
753	254	33·7	247	2·19	113·7	100·5	79·6
763	352	46·2	346	0·92	113·8	100·0	75·1
871	333	38·3	325	—	129·8	113·2	78·7
877	329	37·5	318	3·03	129·2	109·4	79·7
Mean	—	43·8	—	—	—	—	76·6
S.D.	—	8·6	—	—	—	—	3·4

*Blood volume corrected for the salt and protein content of the blood. The volume occupied by 3 g. salt was assumed to be 1 ml. The partial specific volume of the protein was assumed to be 0·74 ml./g.

†Corrected for the salt and protein content of the blood. In those cases where the blood protein was not determined, it was assumed to be 2·0 g./100 ml. The salt concentration was assumed to be 3·0 g./100 ml.

B. *Gastropoda*

(1) *Opisthobranchia*

(*a*) Aplysia californicus. The tectibranch, *A. californicus*, looks and feels like a bag of water. In specimens of a group of twenty-four animals, the mean fluid volume which could be drained off, expressed as percentage of body weight, was 60%, varying from 51 to 71%. It is, therefore, not surprising that analysis of the blood volume by dilution techniques yielded values higher than those of the other molluscs investigated.

A number of different agents were employed for the blood-volume determinations in *Aplysia* and the results obtained are presented in Table 3. Inulin gave a mean blood volume of $79{\cdot}3 \pm 3{\cdot}1\%$; haemoglobin gave $76{\cdot}2 \pm 4{\cdot}4\%$; silver proteinate gave $73{\cdot}1 \pm 6{\cdot}6\%$. In Fig. 1 there is plotted the result of a typical experiment with inulin.

The volume of fluid in which inulin was distributed was greater than that for the other agents. The variability of the data, however, prevents these differences from being highly significant. The difference of the means determined by inulin and by silver proteinate are at the borderline of significance with P of 0·04. The differences between the means of inulin and haemoglobin, and haemoglobin and silver proteinate are not statistically significant. The larger molecules may not penetrate into all of the spaces in which inulin is distributed. The pericardial sac and the lumen of the kidney are the most likely areas, but tests of the inulin content of the fluids of these spaces were not included while the work was in progress, and the animals are not now available for further tests.

Table 3. *Inulin, silver proteinate and haemoglobin volumes, total body water and cellular water of* Aplysia californicus

Animal wet wt. (g.)	Blood vol. (ml.)	Blood vol. as % wet wt.	Blood* water (ml.)	Dry wt. (g.)	Corrected† dry wt. (g.)	Body water as % wet wt.	Cellular water as % wet tissue wt.
				Inulin			
183·5	148	80·9	147	12·7	8·3	93·0	74·1
220·8	178	80·6	176	16·4	11·0	92·6	72·1
237·5	189	79·6	187	15·8	10·2	93·4	77·2
264·5	222	83·8	220	18·4	11·7	93·1	68·9
255·5	192	75·2	190	20·8	15·0	92·0	74·9
270·5	223	82·4	221	18·9	12·2	93·0	71·5
330·7	250	74·2	248	25·7	18·2	92·2	75·9
387·0	301	77·8	298	26·4	17·3	93·2	78·3
Mean	—	79·3	—	—	—	—	74·2
S.D.	—	3·1	—	—	—	—	3·0
				Silver proteinate			
146·0	91	62·3	90	12·7	10·0	91·4	81·3
184·0	144	78·3	143	14·0	9·1	92·4	73·7
236·0	163	69·0	161	16·4	11·5	93·0	83·6
236·5	188	79·5	186	17·0	11·3	92·8	74·7
267·0	200	75·0	198	19·9	13·9	92·5	78·0
286·0	174	62·0	172	23·4	17·8	91·7	82·1
308·8	232	75·1	230	27·8	20·8	91·0	71·1
330·7	256	76·0	253	25·6	18·0	92·3	74·3
333·7	269	80·6	266	24·1	16·1	92·8	73·0
Mean	—	73·1	—	—	—	—	76·9
S.D.	—	6·6	—	—	—	—	3·3
				Haemoglobin			
260·0	188	72·1	186	18·7	13·1	89·0	80·8
270·5	189	70·0	187	18·9	13·2	93·0	83·6
280·0	220	78·5	—	—	—	—	—
290·6	222	76·5	220	21·1	14·5	92·7	77·4
349·0	255	73·0	—	—	—	—	—
554·5	285	80·4	282	25·0	16·5	92·9	74·3
411·0	341	83·0	—	—	—	—	—
Mean	—	76·2	—	—	—	92·4	79·1
S.D.	—	4·4	—	—	—	1·0	3·5

* Corrected for the volume of the salt only.
† Corrected for the weight of the salt only.

Since *Aplysia* has a shell of negligible size it was relatively easy to obtain other data on each specimen of this mollusc. The mean of the total water content of the twenty-one animals was $92{\cdot}4 \pm 1{\cdot}0\%$. The mean of the percentage cellular water differed for inulin, silver proteinate and haemoglobin, the values being $74{\cdot}2 \pm 3{\cdot}0\%$, $76{\cdot}9 \pm 3{\cdot}3\%$, $79{\cdot}1 \pm 3{\cdot}5\%$, respectively

(*b*) Archidoris. The nudibranch *Archidoris* appears to have a more solid structure than *Aplysia*. This may be due in part to the nature of the integument, or perhaps to the body fluids being under greater hydrostatic pressure, for the percentage of total body water in *Archidoris*, $92{\cdot}4 \pm 1{\cdot}3\%$, is the same as that for *Aplysia*.

Table 4. *Inulin volume, total body water and cellular water of* Archidoris *sp.*

Animal wet wt. (g.)	Blood vol. (ml.)	Blood vol. as % wet wt.	Blood* water (ml.)	Dry wt. (g.)	Corrected† dry wt. (g.)	Body water as % wet wt.	Cellular water as % wet tissue wt.
50·6	28·6	56·4	28·3	4·22	3·36	91·7	84·3
51·2	32·3	63·1	32·0	3·44	2·47	93·1	86·5
53·1	30·3	57·1	30·0	3·43	2·52	93·5	88·7
54·6	38·4	70·3	38·0	5·03	3·88	90·8	74·9
57·8	42·5	73·6	42·1	3·85	2·58	93·2	82·1
62·1	44·8	72·2	44·4	4·50	3·15	92·0	80·7
Mean	—	65·5	—	—	—	92·4	82·9
S.D.	—	7·0	—	—	—	1·3	4·4

* Corrected for the volume occupied by the salt only.
† Corrected for the weight of the salt only.

By simple drainage a volume of blood approximately one-fourth the body weight could be obtained readily. This is much less than that from *Aplysia*, but more than that from other molluscs. Consequently, the relatively high percentage blood volume of $65{\cdot}5 \pm 7{\cdot}0\%$, was to be expected. The experimental data for this animal are presented in Table 4, and a typical experimental curve is given in Fig. 1.

The cellular water, $82{\cdot}9 \pm 4{\cdot}4\%$, is a higher value than was obtained for other forms reported, but may only represent a sampling error as the number of animals reported on is small.

(2) *Pulmonata*

(*a*) Achatina fulica. The giant African snail, *A. fulica*, appears to have an effective circulation since the equilibrium time was relatively short. The results of a typical experiment are given in Fig. 1, and the values of body weight, blood volume, etc., are given in Table 5. The blood volumes for eight animals was $40{\cdot}3 \pm 5{\cdot}6\%$, the body water $86{\cdot}4 \pm 3{\cdot}3\%$, and the cellular water $77{\cdot}1 \pm 5{\cdot}0\%$.

Although the animals used in the experiments were supplied with water, the possibility existed that the extent of hydration differed from animal to animal and

Table 5. *Inulin volume, total body water and cellular water of* Achatina fulica

Wet wt. without shell (g)	Blood vol. (ml.)	Blood vol. as % wet wt. without shell	Dry wt. (g.)	Body water as % wet wt. without shell	Cellular water as % wet tissue wt.	Blood protein (g./100 ml.)
41·0	15·8	38·6	6·90	83·2	72·6	0·60
44·5	15·8	35·5	7·10	84·0	75·2	1·63
45·1	23·5	52·2	4·80	89·5	77·8	0·55
45·8	18·5	40·4	8·00	82·4	70·8	0·15
62·3	28·6	45·8	6·95	89·0	79·4	0·36
69·8	24·6	35·2	10·05	85·7	77·7	—
71·8	25·0	34·8	5·50	92·4	88·2	—
73·8	28·6	39·8	11·45	84·6	74·8	—
Mean	—	40·3	—	86·4	77·1	0·45
S.D.	—	5·6	—	3·3	5·0	—

that this might account for some of the variability in the results. Animals which were kept in the laboratory with a restricted amount of water available lost about 10% of their weight the first day. However, the snails appeared to adjust to the desiccation and in some cases lost only about 20% of the original body weight after 9 days without water.

The range in total body water of the animals used in these experiments was about 10%. Nevertheless, the correlation coefficient, *r*, between percentage blood volume and percentage body water was only 0·26, a value which implies no direct relationship in the range tested.

The amount of protein in the blood of five animals was determined by a micro-Kjeldahl method and a mean of 0·46 g. of protein in 100 ml. of blood was obtained. The individual results are given in Table 5.

(*b*) Arion ater. A slug may lose as much as one-fifth of its initial wet weight during a 3–4 hr period. Therefore, care was taken during an experiment to keep the environment saturated with water and to minimize the amount of handling. To detect any changes in weight, the animals were weighed at the beginning and end of an experiment, and only the results for those animals which showed negligible weight losses (less than 5% of body weight) are reported in Table 6.

Table 6. *Inulin volume, total body water and cellular water of* Arion ater

Animal wet wt. (g.)	Blood vol. (ml.)	Blood vol. as % wet wt.	Dry wt. (g.)	Body water as % wet wt.	Cellular water as % wet tissue wt.
10·00	3·45	34·5	—	—	—
11·57	2·88	24·9	—	—	—
11·57	3·95	34·2	—	—	—
11·70	5·00	43·6	—	—	—
11·80	5·88	50·2	—	—	—
13·30	6·33	47·5	—	—	—
14·05	3·37	24·0	—	—	—
12·20	7·02	57·5	1·00	91·8	80·7
13·72	4·71	34·3	1·64	87·0	81·8
21·95	6·46	29·4	2·64	88·0	83·0
22·83	8·00	35·0	3·52	84·5	76·3
43·72	10·88	24·9	8·55	80·3	74·0
Mean	—	36·6	—	86·3	79·0
S.D.	—	10·4	—	3·8	3·4

There was a considerable amount of variability in the blood volumes obtained in the early experiments, the values ranging from 24·0 to 50·2 %. In order to ascertain whether this variability was attributable to the degree of hydration of the animal, dry-weight determinations were made in subsequent experiments. The coefficient of correlation, *r*, between blood volume and body water was found to be high at a value of 0·81 for the later experiments. However, due to the small number of experiments, this *r* is not reliable and the question is not settled.

The mean blood volume for all the animals reported was 36·6 ± 10·4%, the mean total body water was 86·3 ± 3·8%, and the cellular water 79·0 ± 3·4%. The variability in the results may not be extraordinary as the animal is terrestrial and does not possess a protective shell.

C. *Pelecypoda*

Filibranchiata

(*a*) Mytilus californianus. In *M. californianus* after the injection of the test material into the anterior aorta equilibrium was reached in a relatively short time. Data from a typical experiment are presented in Fig. 1, and the results of the blood-volume determinations, as well as other pertinent data, are given in Table 7. The average blood volume is $50{\cdot}8 \pm 7{\cdot}6\,\%$, the total body water is $88{\cdot}9 \pm 0{\cdot}36\,\%$, and the cell water is $79{\cdot}7 \pm 3{\cdot}6\,\%$.

Table 7. *Inulin volume, total body water and cellular water of* Mytilus californianus

Series A

red, vt. shell)	2 Measured blood vol. (ml.)	3 (2/1 × 100) blood vol. as % wet wt.	4 Measured dry wt. (g.)	5 (1 × mean of 13), dry wt. calculated from data of Series B (g.)	6 (1 × mean of 15), dry wt. corrected for blood salt (g.)	7 (2 − 1 % of 2), blood water* (ml.)	8 (1 − [5 + 7]), cellular water (ml.)	9 (8 + 6), wet cell wt. (g.)	10 (8/9 × 100), cellular water as % wet cell wt.
8	21·0	53·8	6·7	4·27	3·68	20·8	13·73	17·41	78·26
8	24·6	61·6	4·9	4·38	3·78	24·4	11·02	14·80	74·46
0	27·3	56·7	8·6	5·28	4·55	27·0	15·72	20·27	77·55
3	37·6	64·5	4·4	6·41	5·55	37·2	14·69	20·24	72·57
5	30·8	41·3	12·3	8·21	7·10	30·5	35·89	42·99	83·48
3	34·8	44·4	14·8	8·61	7·45	34·5	35·19	42·64	82·52
4	39·2	40·4	11·9	9·28	8·01	38·8	36·32	44·33	81·93
6	42·0	44·0	21·9	10·52	9·09	41·6	43·48	52·57	82·70
9	53·2	45·8	18·0	12·78	11·02	52·7	50·52	61·57	82·05
4	60·6	49·5	14·6	13·46	11·63	60·0	48·94	60·57	80·80
n	—	50·8	—	—	—	—	—	—	79·7
	—	7·6	—	—	—	—	—	—	3·56

Series B

11 Measured wet wt. without shell (g.)	12 Measured dry wt. (g.)	13 (12/11 × 100), dry wt. as % wet wt.	14 (12 − 0·03[11/2]), dry wt. without blood salt (g.)	15 (14/11 × 100), corrected dry wt. as % wet wt.
34·20	4·65	13·6	4·13	12·1
35·12	3·99	11·4	3·46	9·8
35·35	3·59	10·1	3·05	8·6
40·93	4·60	11·2	3·98	9·7
43·68	5·04	11·5	4·38	10·0
45·64	4·37	9·6	3·68	8·1
50·92	5·34	10·5	4·56	8·9
55·13	5·79	10·5	4·95	9·0
Mean 42·61	—	11·0	—	9·5

* Blood water is derived from blood volume on the assumptions that the blood protein of *Mytilus* is negligible (less than 0·1 %), and that the volume occupied by 3 g. of salt is 1 ml.
Dry weight is derived from measured dry weight by subtracting the salt content, assumed to be 3 %, of the blood estimated from Series A to be about 50 % of the wet body weight.

It will be noted that two series of experiments are presented. The blood-volume determinations were performed on animals recently brought to the laboratory, and work was completed before a decision had been reached to compute cellular water using a corrected dry weight. No attention had been paid to clearing the gut, a factor which is thought to account for the variability of the dry-weight measurements. Since the blood-volume measurements were technically quite demanding a new series was not performed. Instead, animals which had been kept in the

laboratory for several months were dissected and dried. The corrected dry weights obtained from these animals were then applied to the data derived from the first series. Errors are undoubtedly introduced by this process and it must be kept in mind that the values set forth for cellular water are not more than estimates.

(*b*) Margaritana margaritifera. In *M. margaritifera* the equilibration time was as short as 15 min. Generally, however, 30 min. elapsed before a linear relationship was established. This rapid equilibration may have been the result of the direct injection of the test material into the heart which insures the most rapid distribution through the circulatory system.

Table 8. *Inulin and T-1824 volumes, total body water and cellular water of* Margaritana margaritifera

Wet wt. without shell (g.)	Blood vol. (ml.)	Blood vol. as % wet wt. without shell	Dry wt. (g.)	Body water as % wet wt. without shell	Cellular water as % wet tissue wt.
31·4 (T-1824)	16·6	52·8	4·3	86·3	71·0
(Inulin	16·2	52·6	4·3	86·3	71·6
32·5 (inulin)	13·0	40·0	4·8	85·5	75·5
34·8 (inulin)	13·9	40·0	4·1	88·2	80·3
36·6 (T-1824)	19·0	51·9	4·7	87·2	73·3
(Inulin)	20·0	54·7	4·7	87·2	71·7
38·2 (inulin)	16·6	43·4	4·9	87·2	77·3
38·4 (inulin)	17·8	46·3	3·9	89·9	81·0
39·2 (T-1824)	24·4	62·2	—	—	—
(Inulin)	19·6	50·0	—	—	—
44·0 (inulin)	22·5	51·2	4·2	90·4	80·4
44·5 (inulin)	18·5	41·6	5·7	87·1	78·1
48·6 (inulin)	25·0	51·4	4·6	90·5	80·5
Mean	—	49·0	—	88·0	76·4
S.D.	—	6·0	—	1·5	3·8

The data obtained in a typical experiment are presented in Fig. 1, and the results for all the experiments reported are summarized in Table 8. The blood volume was found to be $49{\cdot}0 \pm 6{\cdot}0\%$. In three of the specimens, successful experiments were performed in which inulin and T-1824 bound to rabbit serum were injected simultaneously. Both substances appeared to measure the same space although they differ in chemical properties and the size of the molecule.

The volume of blood which could be drained from the visceral mass at the end of an experiment ranged from 30 to 50% of the volume determined by the dilution method. In a few experiments the concentration of inulin in the drained blood was determined in order to ascertain whether there was a significant contamination of the drained blood with water. In these cases the concentration of inulin in the drained blood was within the range of concentration of the experimental blood samples.

The total body water was relatively high, $88{\cdot}0 \pm 1{\cdot}5\%$, and the cellular water was $76{\cdot}4 \pm 3{\cdot}8\%$.

Octopoda

D. *Cephalopoda*

(*a*) Octopus hongkongensis. Most of the measurements of the blood volume of the octopus were made with T-1824. It was possible to use this material since the octopus has a high blood protein, about 10 g. of protein per 100 ml. of blood, to which the dye was bound. To substantiate the T-1824 estimate of the size of this compartment, colloidal HgS was also used as a test material. The two substances appear to measure the same fluid compartment, but differ in the rate of loss from the circulating fluid, the HgS being lost at the slower rate. Representative curves of experiments with these two substances are given in Fig. 1. In Table 9 are summarized the data for the octopus, the blood volume being $5{\cdot}8 \pm 1{\cdot}0\%$. The percentage total water was determined for three animals and was found to be $82{\cdot}5\%$ of the body weight. Using this value, the cell water is approximately 77% of the wet tissue weight.

Table 9. *Inulin volume, T-1824 volume and* HgS *volume of* Octopus hongkongensis

Animal wet wt. (g.)	Test material	Blood vol. (ml.)	Blood vol. as % wet wt.	Extracellular vol. (ml.)	Extracellular vol. as % wet wt.	Extracellular equilibration time (min.)
7,730	T-1824	588	7·6	—	—	—
4,550	T-1824	217	4·8	—	—	—
10,430	T-1824	624	6·0	—	—	—
6,360	T-1824	374	5·9	—	—	—
12,955	T-1824	759	5·9	—	—	—
22,500	T-1824, raffinose	1272	5·6	5460	24·3	165
8,640*	T-1824, inulin	492	5·7	1612	18·7	210
8,750	T-1824, inulin	492	5·6	1776	20·3	225
14,900	T-1824, inulin	884	5·9	4550	30·5	180
8,520	T-1824, inulin	649	7·6	3450	40·4	85
8,640*	HgS	374	4·3	—	—	—
14,780	HgS	943	6·4	—	—	—
8,175*	HgS, inulin	375	4·6	1835	22·5	125
19,550	Inulin	—	—	5970	30·6	195
4,890	Inulin	—	—	1775	36·5	120
Mean	—	—	5·8	—	28·0	—
S.D.	—	—	1·0	—	7·3	—

* Same animal.

In one experimental animal it was possible to make several measurements. In the first experiment, T-1824 as used with a resultant blood volume of $5{\cdot}7\%$. Three days later, a repeat determination was made with colloidal HgS, and a blood volume of $4{\cdot}3\%$ was obtained. A month later, another measurement was made with HgS with a value of $4{\cdot}6\%$ being obtained. The animal seemed perfectly normal, the only change noted was a loss of weight from 8640 to 8175 g., although the animal had continued to eat regularly.

For comparison with the volumes obtained with T-1824 and HgS, the volume of

fluid in which inulin was distributed was measured. In some cases the inulin was administered simultaneously with the other agents, and at other times independently. The average value obtained for the inulin space was 28·0 ± 7·3%. The experimental data are summarized in Table 9 and a typical experiment is shown in Fig. 1.

There has been anatomical evidence for a closed circulatory system in cephalopods, but experimental verification has been lacking. From the results that have been obtained, it is believed that inulin measures the total extracellular fluid, while T-1824 and HgS measure the blood volume. Since the inulin space was 28·0% and the blood volume 5·8%, the tissue fluid compartment of the octopus is approximately 22% of the wet tissue weight.

The coefficient of correlation, r, between the time necessary for equilibrium and the size of the extracellular space was −0·71. In testing the significance of this value a P of 0·05 was obtained even though the number of samples was small. Consequently, it appears likely that an inverse relationship exists between the two values.

DISCUSSION

The results of the determinations of blood volume in representative molluscs show a wide range of values within the phylum from 5·8% in *Octopus* to about 75% in *Aplysia*. Even if the total extracellular fluid of *Octopus*, about 28%, is considered to be the better figure for comparison with the representatives of the other classes, the range is still a very wide one and illustrates great diversity within the phylum.

The low blood volume of 5·8% reported for *Octopus* is obviously a different order of magnitude from any other of the forms studied. Only in this species was there a great difference in the volumes of fluid in which materials of different molecular size were distributed. The dye T-1824, which complexes with the protein of the blood and colloidal HgS (a material of colloidal particle size), appeared to measure the blood volume, while inulin distributed in a considerably larger space, probably the extracellular fluid. Since on a percentage basis the volume of the extra-cellular fluid of the *Octopus* is lower than the blood volumes of the other forms, it follows that a closed circulatory system containing a high concentration of respiratory pigment separated from a tissue fluid compartment may meet greater metabolic demands than an open circulatory system with a larger total volume of fluid.

Robertson (1953) reported a value of 33% for the extracellular fluid of the cephalopod *Sepia*, but with the reservation that this might be an overestimate due to the possible entrance of the test material, sucrose, into muscle cells. It was noted in the course of the work reported here that there was an inverse relationship between the size of the extracellular space measured by inulin and the time required for equilibration. This observation would appear to indicate differences in permeability of tissues from animal to animal, so that a high permeability and a rapid attainment of equilibrium would also permit the entrance of inulin into other spaces, and a higher apparent extracellular fluid volume. A part of the variability of the results with this substance may rest on these unexplained differences in permeability. Penetration of inulin into other spaces will be discussed later in this paper.

The groups which show total blood volumes next larger in size to those of the

cephalopods are the pulmonate gastropods. Since both *Achatina* and *Arion* have open circulatory systems and are terrestrial, the differences expected between their average blood volumes, 40·3 and 36·6%, respectively, would not be very great. When the *t* test was applied to the data for these forms, it was found that the difference was not large enough to be statistically significant given the variability encountered in the measurements.

The mean blood volume of 44% obtained for the most primitive of the animals included in this study, *Cryptochiton*, is somewhat larger than those of the pulmonates. This value is statistically significantly different from that of *Arion*, but not from that of *Achatina*.

In order of increasing blood volume, the next species encountered are in the class Pelecypoda. *Mytilus*, with a blood volume of 51%, and *Margaritana* at 49%, show a striking similarity in spite of one being a marine and the other a fresh-water animal. Since the animals are similar in size, shape and morphology, perhaps this should be expected. The difference between the means of these two forms is not statistically reliable, but the blood volumes of these animals are significantly different from all the others reported here.

The results obtained with *Mytilus* and *Margaritana* confirm the findings of Potts (1954) who determined the inulin space of the fresh-water pelecypod *Anodonta cygnea* to be 55% of the wet weight without shell. In view of this similarity in blood volume of one marine and two fresh-water pelecypods, and the general agreement of these results with those on the other molluscan forms, it appears likely that the value of 8% of the wet tissue weight reported for two other fresh-water pelecypods by Prosser & Weinstein (1950) may be too low. Low values might be obtained under a number of different circumstances. Because they injected through a hole in the shell, without visual confirmation that the tip of the needle lay within the heart, it is possible that the material was injected into some fluid other than the circulating blood. On the other hand, we have noted that too rapid an injection of test material into the circulatory system resulted in impairment of heart action. It is possible that in their experiments the injected material entered the blood, but was mixed with a relatively small part of the total volume of the circulatory system in the 30 min. of the experiment. As confirmatory evidence for the low value Prosser & Weinstein (1950) cite the recovery from the heart at the conclusion of the experiment of fluid amounting to 96% of the volume measured by T-1824. In none of the species studied in our work was it possible to recover quantities of this order of magnitude, and it is our opinion that such a recovery is rather an indication of error than a satisfactory accounting for the volume of blood of the animal.

The largest blood volumes were found in the representatives of the opisthobranchs, *Archidoris* having a mean value of 65% and *Aplysia* one of approximately 75%. The difference between the means is statistically significant and the blood volumes of these animals are reliably different from those of all the other animals investigated. These blood volumes are so large that it must be inferred that the blood is meeting not only the ordinary demands upon a circulatory system, but some other requirement of these animals. The idea that water may serve as a

structural element goes back at least to the arguments for the gonocoel theory advanced by Hatshek (Goodrich, 1945). The representatives of the group studied in this work demonstrated a very definite control over the distribution of the blood in the body. It will be recalled that until a multiply perforated tube was inserted in the haemocoel for more than half the body length every opening in it could be closed off by contraction of the animal's body wall. The animal demonstrated fine control of its body form in other respects. A large *Aplysia* depleted of much of its supply of blood soon took on a normal form and could be recognized by inspection as having been a larger animal only by the relatively large size of the ctenidium. It may also be pointed out that this group feeds on marine plants which are relatively voluminous for the amount of dry digestible nutrients. On this poor diet, a large body size would be perhaps of competitive value, and a large volume of water would represent a cheap way of attaining size without increasing the metabolic requirements exorbitantly.

The differences in blood volume obtained with various substances in *Aplysia* may serve to illustrate the statement of Potts (1954) that it is likely that inulin determines not only the circulating blood volume, but also the pericardial and renal coelomic space in *Anodonta*. Measurements with silver proteinate gave 73%, with inulin, 79%—a difference which amounts to 6% of the wet body weight. This might be due to failure of the silver proteinate to penetrate into these spaces. The relative size of this error would then vary from species to species, depending upon the size of these spaces compared to the blood volume.

Further evidence for the penetration of inulin into other spaces is found in the experiments on excretion in the *Octopus* (Harrison, 1954) and in *Achatina fulica* (Martin, Harrison & Stewart, 1953). In these forms it is known that inulin penetrates the renal space, and appears after a lag period in the urine in approximately the same concentration as that found in the blood. Robinson & McCance (1952) have stated well the difficulties in using single injections of inulin in mammals when excretion is rapid. In contrast, the excretion by molluscs is very slow. Loss of inulin by this route may be compensated by the extrapolation of the dilution curve back to the time of injection. However, the entrance of inulin into the pericardial and renal spaces may account in part for the early, non-linear aspect of the dilution curve. In view of all the evidence at hand it may be well to regard all the volumes measured with inulin as somewhat greater than the true blood volume.

In addition to the differences in blood volume from species to species, there was also a considerable variation in the amount of time necessary for equilibration. This is probably due to a number of factors, including the region of injection, the effectiveness of the circulatory system, and the amount of movement of the body. In an inactive form such as *Cryptochiton*, the injection had to be made into the haemocoel, and slow equilibration might be expected. In contrast, when the injection was made directly into the heart and the animal showed considerable bodily activity, as in *Margaritana*, the equilibration would be rapid. Slow equilibration made necessary a longer extrapolation with a consequent multiplication of any errors due to differences in permeability.

Another aspect of the data is yet to be considered. The total body water ranged in value from 82·5 to 92·4%. The *t* test was used to determine the significance of the differences in the mean total water contents. The mean body water of *Aplysia* and *Archidoris*, both 92·4%, is reliably different from all of the others. The difference between *Mytilus* at 84·6% and *Octopus* at 82·5%, is significant, while that between *Mytilus* and *Margaritana*, 88%, is probably significant—$P = 0{\cdot}02$. None of the remaining comparisons was reliably different.

The parameter to be considered in conclusion is the cellular water. For each species a computation of the percentage of cellular water has been made and the data presented in the tables for individual animals. This computation can be obtained readily, since for most molluscs there is an open circulatory system and the shell, if present, is easily freed from the body tissues.

The range of values of cellular water is from 76·4 to 82·9% and differences between the values are hardly significant. Possible sources of error have been pointed out to which it should be added that no correction was applied to the data for the connective tissue of the animals. Even though these values should be considered only as approximations, it is of interest to compare them with some of those reported in the literature. The water content of the tissues of molluscs is not well known. Balland's (1898) values are those generally cited, but this author made no effort to distinguish extracellular from intracellular water, and other reports on molluscan tissue suffer from the same difficulty. On the other hand, Ephrussi (1933), working with echinoderm eggs and larvae, corrected for extracellular water in his determinations. In 40 hr. larvae of *Paracentrotus lividus* he found the cellular water amounted to 78·8% of the total wet weight. In the vertebrate group of animals it is only in recent years that the results of computations of the water content of mammalian cells have been in general agreement from laboratory to laboratory. Chanutin & Ludewig (1939) calculated the intracellular water of rat muscle fibres to be 73·2%. Lowry & Hastings (1942) are in essential agreement, their value being 74%. These figures are further confirmed by a 74·2% value for dog muscles (Muntwyler, Mellors, Mautz & Mangun, 1940) and of 75·1% for cat muscle which can be calculated from the data of Crismon, Crismon, Calabresi & Darrow (1943). But most of these values rest on the debatable assumption that there is no chloride in the cells and so the values may also be considered to be estimates. Computations for certain other mammalian tissues are available, but since these computations only demonstrate the expected variation from mammalian tissue to tissue, they will not be cited here. The water content of the various living cells reported here is, as might be expected, quite comparable with the others.

One of the objectives of this study was to examine the normal variability in blood volume in representatives of this phylum of animals. A number of factors have been discussed which may be sources of error in the results. However, within one species, these errors should be reasonably consistent, and the range of variability of blood volume is thought not to be due entirely to technical faults, but as reflecting the inability of these animals to regulate their blood volume with a high degree of stability.

SUMMARY

1. Individuals of *Cryptochiton stelleri*, as representatives of the class Placophora, distributed inulin in blood volumes yielding a mean of 43·8% of the wet body weight without shell. Mean cellular water was estimated to be 76%.

2. The two opisthobranch gastropods examined were found to have very large mean blood volumes. *Aplysia californicus* distributed haemoglobin in 76·2% and mild silver proteinate in 73·1% of the wet body weight. Inulin was distributed in a significantly larger space averaging 79·3% of the body weight, which probably included pericardial and renal spaces. *Archidoris* sp. distributed inulin in 65·4% of the wet body weight. Mean cellular waters were 74–79% in *Aplysia* and 83% in *Archidoris*.

3. Two pulmonate gastropods were studied with inulin which was distributed in a mean space 40·3% of the wet body weight without shell of *Achatina fulica*, and 36·6% of the wet body weight in *Arion ater*. The computed cellular waters were 77 and 79%, respectively.

4. Excellent agreement was shown between a marine pelecypod, *Mytilus californianus*, and a fresh-water pelecypod, *Margaritana margaritifera*, of similar body size and form. Inulin in the former was distributed in 50·8%, and in the latter inulin and T-1824 in 49% of the wet body weight without shell. The cellular water contents were 80 and 76%, respectively.

5. In a single representative of the Cephalopoda—*Octopus hongkongensis*, it was possible to demonstrate with T-1824 and with HgS a blood volume averaging 5·8% of the wet body weight, constituting a fluid space distinctly different from the tissue fluid space. Inulin was distributed in the entire extracellular space amounting to 28% of the wet body weight. The cell water which was calculated from the mean values obtained was 77%.

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SOARING AND GLIDING FLIGHT OF THE BLACK VULTURE

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INTRODUCTION

In 1950 Raspet published an interesting paper giving the glide performance of the black vulture (*Coragyps atratus atratus*). The sinking speed of several birds of this species was measured by flying in formation with them in a light sailplane and estimating the relative rate of descent. Prior knowledge of the sinking speed of the sailplane then enabled the performance of the bird to be determined.

It was discovered that the bird had two distinct types of gliding flight, which were designated soaring and gliding. When soaring the bird circles in a rising current of air, usually a thermal, and remains aloft without flapping its wings; to do this a low sinking speed in still air is required. When gliding, on the other hand, the bird travels over ground possibly looking for food or possibly travelling from thermal to thermal. To do this a low rate of sink combined with a comparatively large airspeed is required, because it may be necessary to penetrate into the prevailing wind. It appears that the black vulture, in common with other land-soaring birds, satisfies these two requirements by altering the geometry of its wings. When soaring the primary tip feathers may be spread and the leading edge of the wing is bent forward slightly; when gliding the primaries are closed and bent backwards. Typical wing planforms are reproduced from Aymar (1935) in Fig. 1. Some good photographs of these birds can be seen in the works of Aymar (1935), Storer (1948) and Barlee (1953). When soaring with a wind crossing the thermal the birds appear to combine both types of flight; wings fully open on the downwind part of the circuit and slightly closed when completing the turn into wind. In this way the bird manages to remain within the thermal at zero or negative sinking speed.

The opening of the wing feathers clearly increases the span of the wing and thereby reduces the trailing vortex, or induced drag, but probably at the expense of increasing the profile drag slightly. The minimum sinking speed is proportional to $C_{D_0}^{\frac{1}{2}}/b^{\frac{5}{4}}$, where C_{D_0} is the profile drag coefficient and b is the wing span; thus it is apparent that an increase of b, even with a similar proportionate increase of C_{D_0}, is beneficial. For fast gliding, on the other hand, the vortex drag, which decreases with the square of the speed, becomes relatively less important than the profile drag, which increases with the square of the speed; then it is beneficial to reduce the area of the wing by reducing the wing span.

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The purpose of the present paper is to re-examine in more detail Raspet's measurements on the black vulture, to analyse the effect of opening the tip feathers and, in the light of this, to suggest the reason for the existence of slotted primary feathers on land-soaring birds.

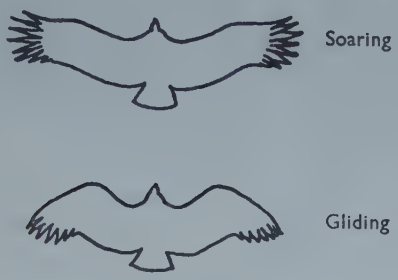

Fig. 1. Sketches of wings of black vulture.

RE-EXAMINATION OF RASPET'S MEASUREMENTS

Gliding flight. For this type of the flight the geometry of the wing planform is constant or nearly so. Thus, the rate of sink in still air, V_s, may be related to the equivalent air speed, V_e, by the usual performance equation

$$\frac{WV_s}{V_e\sqrt{\rho_0/\rho}} = C_{D_0}(\tfrac{1}{2}\rho_0 V_e^2)S + \frac{1}{\pi e b^2}\frac{W^2}{(\tfrac{1}{2}\rho_0 V_e^2)},$$

where W is the all-up weight,

ρ is the prevailing air density,

ρ_0 is the standard sea level density (1·23 kg./cu. m.),

S is the wing area,

b is the wing span,

e is the Oswald span efficiency factor.

Thus
$$V_s V_e = \frac{1}{2}\frac{\rho_0^{\frac{3}{2}}}{\rho^{\frac{1}{2}}}\frac{S}{W}C_{D_0}V_e^4 + \frac{2W}{\pi e b^2 \rho^{\frac{1}{2}}\rho_0^{\frac{1}{2}}}.$$

(Finding the minimum value of V_s from this expression and assuming S to be proportional to b, yields the formula for minimum rate of sink quoted in the introduction.)

Raspet's data for gliding flight are plotted as V_sV_e against V_e^4 in Fig. 2 and the measurements are seen to lie accurately on a straight line. Assuming

$W = 2{\cdot}32$ kg.

$\rho = 1{\cdot}19$ kg./cu. m.—estimated to be prevailing at the time of flight.

$e = 90\%$—an estimated value for the gliding planform (Fig. 1),

the intercept of the line with the V_sV_e axis yields $b = 1{\cdot}12$ m. which is about 20% less than the value in the soaring flight configuration ($b = 1{\cdot}44$ m.). Observation of the black vulture does indicate a change of wing span of this order (see Fig. 1).

Assuming that the wing area is also 20% less than that of the soaring configuration, the slope of the line gives $C_{D_0} = 0{\cdot}0064$,

and, with a ratio of wetted area to wing area of about 2·5, the drag coefficient based on wetted area is 0·0026. The range of Reynolds's number based on mean wing chord for the gliding flight is $2{\cdot}9 \times 10^5$–$4{\cdot}4 \times 10^5$ and the drag coefficient of a smooth flat plate moving parallel to its plane at these Reynolds numbers varies from 0·0025 to 0·0020 if the boundary layer flow is laminar, and from 0·0059 to 0·0053 if it is turbulent. Hence, as noted by Raspet, the bird achieves a drag only slightly in excess of the value for a flat plate with laminar flow.

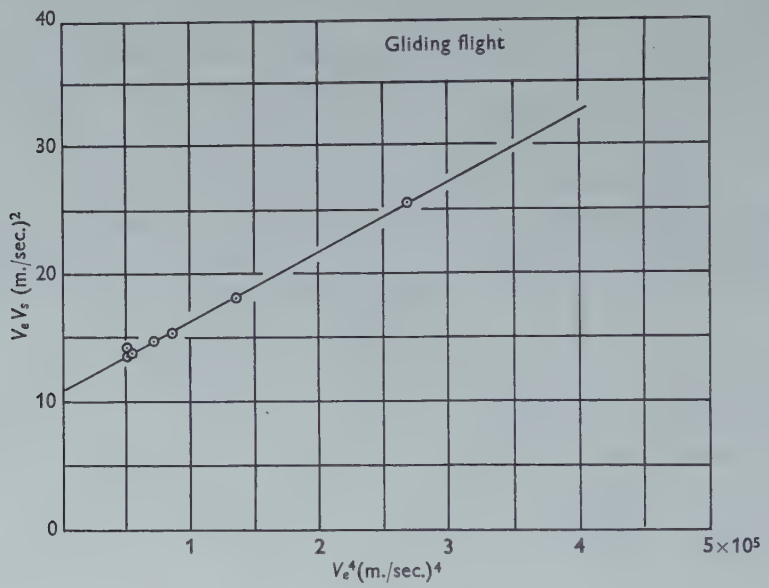

Fig. 2. Gliding flight of black vulture.

Soaring flight. The measurements for soaring flight are presented in the form $V_s V_e$ against V_e^4 in Fig. 3. This figure includes the gliding curve of Fig. 2 and also the curve obtained by assuming a 20% increase of span and wing area, combined with a 20% increase of drag coefficient. It is seen that the results at low air-speeds lie fairly well on the latter curve, that one point lies on the glide curve and that a second point is well above both curves. For this second point, the bird is travelling at low incidence with highly cambered wings, and thus the drag coefficient is increased due to separation of the airflow from the under surface. In addition the drag may also be increased by aerodynamic distortion of the primary feathers when in the soaring configuration. Thus, the drag coefficient may well be increased by a factor of something like 100% and account for the increased rate of sink which was measured.

It is seen, therefore, that the results are consistent with plausible changes of wing geometry and drag coefficient with forward speed.

The results do not support the contention of Fisher (1946) and Raspet (1950) that, from the viewpoint of vortex drag, the effective aspect ratio of a wing with open tips is higher than the purely geometric value. This is investigated further in the

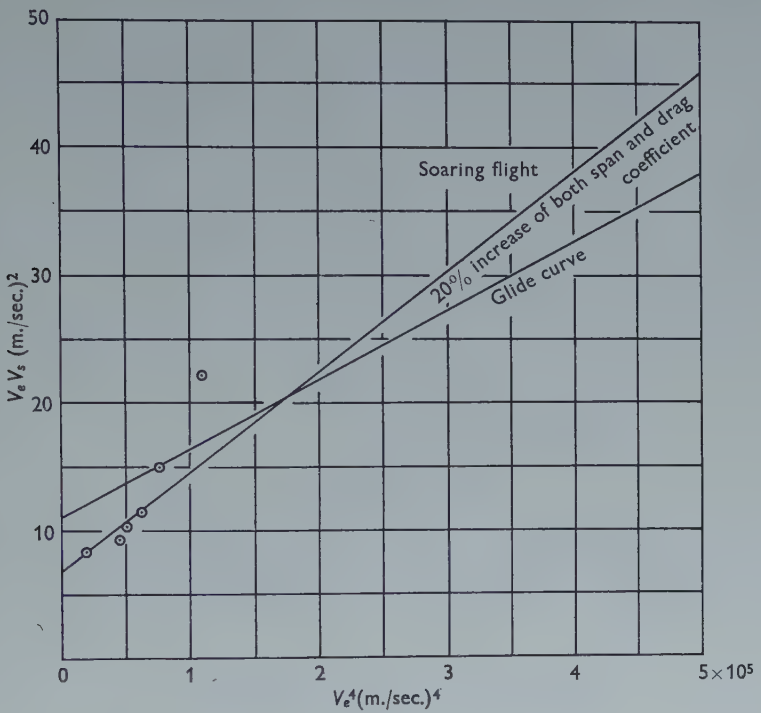

Fig. 3. Soaring flight of black vulture.

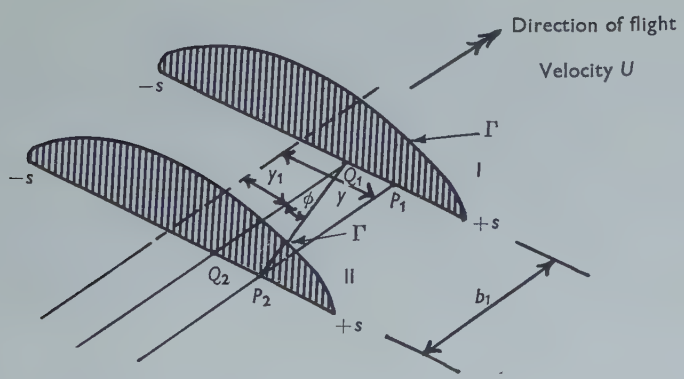

Fig. 4. Three-dimensional sketch of bound vortices in tandem.

appendix of the present paper where it is shown, within the framework of certain simplifying but, nevertheless, plausible assumptions, that the vortex drag of a series of equally loaded tandem wings is identical with that of the single wing obtained by closing the gaps. There appears to be no support therefore for the theory that the vortex drag is reduced by opening the primaries.

SUMMARY

1. The soaring and gliding performance of the black vulture has been analysed and the following conclusions are drawn.

2. The wing span of the bird is altered in flight so that it may perform two tasks efficiently. First, that it may soar in rising currents of air for which a low sinking speed and thus a large wing span are required. Secondly, that it may penetrate into wind without undue loss of height for which a reduced wing area is desirable. Adjustment of the wing geometry towards the optimum soaring configuration is achieved by bending forward and opening the primary tip feathers.

3. Since the airflow readily separates from the flat primary feathers at high angle of attack, these feathers, which are emarginated, are parted to form slots. The alula also presumably assists in delaying the flow separation over the primaries.

4. It is unlikely that the opening of the primaries reduces the vortex drag.

I am indebted to Dr August Raspet for giving me access to unpublished information and for commenting upon this paper.

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APPENDIX

The trailing vortex drag of tandem wings

Consider two wings I and II in tandem. Using conventional lifting line theory (see, for example, Glauert (1948)) the wings are each replaced by a bound, line, vortex.

In Fig. 4 the loading or, what is equivalent, the circulation is as shown. It is assumed to be the same on each wing.

In order to determine the drag of the tandem wings, the induced down-wash due to the bound vortices and the trailing vortices shed from them is examined.

On Wing I the induced downwash velocity at Q_1 due to the infinitesimal trailing vortex shed at P_1 is $\dfrac{-d\Gamma}{4\pi(y-y_1)}$.

The downwash at Q_1 due to the trailing vortex leaving the wing at P_2 on Wing II, is $\dfrac{-d\Gamma(1-\cos\phi)}{4\pi(y-y_1)}$, where $\phi = P_2\hat{Q}_1Q_2$,

and due to the infinitesimal portion dy of bound vortex at P_2, is

$$\frac{-\Gamma dy\cos^3\phi}{4\pi b_1^2}$$

Thus, the total downwash at Q_1 is

$$w_1 = \int_{-s}^{+s} \frac{-d\Gamma}{4\pi(y-y_1)} - \int_{-s}^{+s} \frac{\Gamma \cos^3 \phi dy}{4\pi b_1^2} - \int_{-s}^{+s} \frac{(1-\cos\phi)\, d\Gamma}{4\pi(y-y_1)}.$$

Similarly, the total downwash at Q_2 is

$$w_2 = \int_{-s}^{+s} \frac{-d\Gamma}{4\pi(y-y_1)} + \int_{-s}^{+s} \frac{\Gamma \cos^3 \phi dy}{4\pi b_1^2} - \int_{-s}^{+s} \frac{(1+\cos\phi)\, d\Gamma}{4\pi(y-y_1)}.$$

Thus

$$w_1 + w_2 = -\int_{-s}^{+s} \frac{d\Gamma}{\pi(y-y_1)}.$$

The total trailing vortex drag of both wings

$$= \int_{-s}^{+s} \rho(w_1+w_2)\, \Gamma dy,$$

where ρ is the air density

$$= \int_{-s}^{+s} \rho 2\Gamma \left[-\int_{-s}^{+s} \frac{2d\Gamma}{4\pi(y-y_1)} \right] dy_1.$$

This is identical with the vortex drag of a single wing with the same distribution of circulation and the same *total* lift. In other words splitting a wing to produce two tandem wings does not alter the vortex drag. Furthermore, the above result is clearly true for any number of equally spaced wings in tandem as long as the loading is distributed equally between them.

IN VITRO CULTURE OF EMBRYOS IN THE SILKWORM, *BOMBYX MORI* L.

I. CULTURE IN SILKWORM EGG EXTRACT, WITH SPECIAL REFERENCE TO SOME CHARACTERISTICS OF THE DIAPAUSING EGG

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(With Plate 9)

It is generally believed that the diapausing egg of the silkworm contains a substance which is transmitted from the mother and has an inhibitory action upon the development of the embryo. Watanabe (1924) was the first author to explain the phenomenon of diapause in the silkworm egg by postulating this substance which he called 'inhibitory substance', and almost all subsequent authors in Japan have accepted this assumption. It is not unreasonable to suppose that the 'inhibitory substance', which reveals its action in the egg after deposition, has some connexion with the diapause factor (hormone) secreted by the suboesophageal ganglion of the mother (Hasegawa, 1951, 1952; Fukuda, 1951), but nothing is known about the real nature and action of the diapause factor (Fukuda, 1955).

On the other hand, Umeya (1937*a*, *b*, 1938, 1939, 1952) insists that in the diapausing egg, although the embryo is always ready to grow, some unfavourable condition of the yolk surrounding the embryo inhibits its development beyond a certain stage during diapause, while Miura (1932*a*, *b*, 1938) claims that not only the yolk, but also the embryo itself remains inactive in the diapausing egg. The author has investigated the culture of the silkworm embryo *in vitro* with the object of elucidating these problems by separating embryos from their native yolk and culturing them with experimental media. This paper deals with the results of culturing embryos with silkworm egg extracts.

MATERIAL

Diapausing, non-diapausing, acid-treated, chilled, and chilled-and-acid-treated eggs of various silkworm strains were used as materials.

Diapausing eggs. In diapausing eggs, yolk cells begin to migrate towards the periphery of the egg about 2 days after deposition, and with the completion of this movement in 2 weeks or so (Takami, 1953, 1954*a*) the eggs reach a resting stage (Nittono, 1955; Nittono & Takeshita, 1953). This stage is cited as full diapause in this paper. Such a fully diapausing egg, whose embryo is shown in Pl. 9, fig. 3,

cannot be activated until it has over-wintered. Watanabe (1931) reported that diapausing eggs, if kept at 25–27° C. continuously, usually died after about 190 days, only very few eggs being able to hatch. It is probable that activation of the eggs is not checked at these temperatures, but is retarded so long that most of the eggs die before the completion of activation (Kutsukake, 1954). The eggs used in the present work were widely different in age, ranging from 1 day to several months at 25° C., with corresponding differences in the degree of diapause. Embryos of 1-day-old diapausing eggs were at the broad germ-band stage (Pl. 9, fig. 1).

Non-diapausing eggs. Non-diapausing eggs are also at the broad germ-band stage 20–24 hr. after deposition, and reach the beginning of appendage formation at about the 40th hr. at 25° C. In the present study, eggs 20–30 hr. old were used.

Acid-treated eggs. Diapausing eggs at about the 20th hr. can easily be changed to non-diapausing eggs artificially by immersing in hydrochloric acid (specific gravity 1·075, 46° C., 5 min.). Eggs thus treated were rinsed in running water for about 10 min. and used for experiments before appendage formation.

Chilled eggs. These were eggs which had been stored at 5° C. from the 20th or 50th hr. for different periods until use. The eggs are sufficiently activated to start growth at the end of about 90 days' cold storage, showing good hatchability without any acid treatment if incubated at 25° C.

Chilled-and-acid-treated eggs. One month's cold storage at 5° C. from 50 hr. after deposition is usually insufficient to activate diapausing eggs completely, but during cold storage the eggs become sensitive to acid treatment, and can be changed to non-diapausing ones by a supplementary treatment with somewhat stronger hydrochloric acid (specific gravity 1·10, 47° C., 5 min.) than in the case of the above mentioned acid-treated eggs. The eggs thus treated are called chilled-and-acid-treated eggs in this paper.

METHOD

It is convenient to use silkworm eggs laid on paper. Pieces of a suitable size for handling were cut out from the egg paper bearing the eggs, washed in 5% formalin for 5 min., rinsed in distilled water, and after immersion in 94% ethyl alcohol (two changes of 2–3 min. each) sufficiently dried to ensure adhesion of the eggs. The fixing of eggs on the paper is desirable to facilitate subsequent operations. After the drying, the chorion of the egg was carefully cut open with a sharp needle under a dissecting microscope, and the piece of egg paper transferred to salt solution (Table 1) in a watch-glass in which embryos were dissected out of the eggs with needles and pipettes. An embryo thus obtained was freed from yolk cells adhering on it by repeated gentle sucking in and out of a small-bore pipette, if necessary with additional use of needles, and then put on the underside of a cover-slip by means of the pipette. The salt solution which accompanied the embryo to the cover-slip was replaced with a drop of the culture medium described below. The cover-slip was sealed on a depression slide with melted paraffin, making a hanging-drop culture which was incubated at 25° C. It was necessary to add sufficient culture medium to cover the explanted embryo, because the explant cultured with

insufficient medium always swelled and died without showing any sign of development. All these procedures after immersion in alcohol were carried out under aseptic conditions.

Salt solutions and culture media. The object of the present work was to find out if there is any difference between diapausing and non-diapausing embryos in their growth response to the yolk. By the 'yolk' is meant the silkworm-egg contents which can be sucked up with a small-bore pipette, so that it may contain mostly yolk together with other egg inclusions. The eggs supplying yolk were used after the same treatments, washing in formalin, immersion in alcohol and sufficient drying, etc., as those which supplied the embryos. The response of the embryos to each component of the yolk is an ultimate object of this study, but not the problem of this paper.

Table 1. *The salt solutions used for dissecting eggs and preparing culture media*

	Solution 1	Solution 2
(A) NaCl	7·5 g.	7·5 g.
KCl	0·5 g.	0·5 g.
$CaCl_2 . 2H_2O$	0·2 g.	0·2 g.
$NaH_2PO_4 . 2H_2O$	0·05 g.	0·05 g.
Glucose	—	0·2 g.
Distilled water	750 ml.	750 ml.
(B) $NaHCO_3$	0·05 g.	0·05 g.
Distilled water	250 ml.	250 ml.

A and B are sterilized separately, and mixed in this ratio after cooling. pH 6·7–7·0.

An extract of eggs, used as the culture medium, was made by mixing the yolk (1 part) thus obtained with the salt solution (2 parts) presented in Table 1. In the earlier experiments this mixture was allowed to settle for several minutes after stirring, without being centrifuged, and a comparatively clear part of it was sucked up as the extract for culturing embryos. It was, however, inevitable that the egg extract thus prepared often contained (in addition to abundant suspended particles which would gradually settle on the explant) embryonic fragments which were able to grow and lead to confused development (Pl. 9, fig. 6). The later experiments, therefore, were carried out after eliminating these materials by centrifuging the extract in a glass-tube (2·5 × 30 mm.) for 10 min. at 1000 r.p.m. (the extract from diapausing eggs older than 3 days) or at 1500 r.p.m. (the other extracts). By such centrifugation the extract was fractionated into three layers, that is, a sharply limited upper, lower and rather clear middle layer. The last occupied the greater part of the tube, showing a gradual change in clearness from one end to the other, and could be used as a suitable culture medium. In the diapausing egg, as a result of the migration of yolk cells, the aqueous phase begins to be segregated from the non-aqueous phase about 3 days after deposition. This becomes more distinct with age, and, accompanying this change, fractionation becomes easier in the older eggs. It was for this reason that the fractionation was carried out at different speeds of

rotation according to the age of the eggs. Since the results of cultures with the centrifuged extract showed the same tendency as those obtained with the non-centrifuged extract so far as the growth response of embryos was concerned, most of the experiments with the former will be presented below, together with some supplementary data from cultures with the latter.

Salt solution 1 may be somewhat hypotonic, causing swelling of embryos. However, even these swollen embryos were able to develop in the extract prepared with this solution, and when they started development in the extract their normal shape was restored within a day. In solution 2, swelling of embryos was not observed. Though the two solutions differed from each other in the inclusion of glucose, the two kinds of egg extracts prepared with these solutions showed similar effects on culturing embryos, apart from the swelling of embryos in solution 1. Physiological solutions with a higher ratio of potassium to sodium were recommended for lepidopterous insects, in the course of the work, by many authors (Barsa 1954, Ishikawa & Miyoshi, 1956, Wyatt, 1956); but since the author had started the experiment with the solutions described, the formula was kept unchanged throughout the series.

Embryos never grew in the salt solutions without addition of the yolk, though the change in shape from the broad germ-band to the slender embryo (Pl. 9, fig. 2) was observed in solution 2 as well as in the yolk extract.

Observation. Cultures were continued, without renewal of the medium, usually for 7–10 days during which development of the explants was examined with a low-power microscope every day, and at the end, examinations were made in detail after rinsing the embryos in the salt solution to remove adhering sediment and, if necessary, tearing away the amnion.

In many cases it is rather difficult to judge whether diapausing embryos in culture have really started development or not. For the sake of accuracy, therefore, appendage formation was taken as the criterion of development in the present work, because this was a development stage (Pl. 9, fig. 4) which was never reached by normal diapausing embryos. The following descriptions are based on about 300 and 500 cultures tested in 1955 and 1956, respectively.

RESULTS

Non-diapausing and fully diapausing embryos. When cultures were made with the extract from non-diapausing eggs, explanted embryos of non-diapausing eggs showed a noticeable difference in development from those of fully diapausing ones (Table 2). In the former, segmentation of the ectoderm, formation of the appendages and invagination of the stomodaeum and of the proctodaeum, etc., proceeded until the stage just before the revolution, bristle formation and mandibular pigmentation being often reached (Pl. 9, fig. 5). In the latter, the development, if started, was only partial, very rarely going on as far as appendage formation. Most of the thirty-six cultures of fully diapausing embryos shown in Table 2 resulted in swelling (a sign of failure in development) within a few days, and none reached appendage formation.

In cultures in which appendage formation of embryos was reached marked spreading of the amnion (Pl. 9, fig. 7) and migrating out of mesoderm cells were common, often being accompanied with hernia of the proctodaeum.

In cultures made with the extract from fully diapausing eggs, even non-diapausing embryos, which grow actively in the extract from non-diapausing eggs as mentioned above, showed a markedly restricted development, only 36·4% (8/22) of them reaching appendage formation. All the fully diapausing embryos which were tested failed to develop in this extract.

Table 2. In vitro *culture of non-diapausing and fully diapausing embryos with the egg extract from non-diapausing eggs* (*solution* 2)

Embryo	Non-diapausing			Fully diapausing	
	Over-wintered	Chilled	Acid treated	More than 1-month-old	25-day-old
Egg extract	Over-wintered, non-centrifuging	Chilled, non-centrifuging	Acid treated, centrifuging	Chilled, non-centrifuging	Acid-treated centrifuging
No. of cultures	6	25	20	25	11
Appendage formation No.	5	25	18	0	0
Appendage formation %		94·1		0	

Table 3. In vitro *culture of 1–2 day diapausing embryos* (*solution 2, centrifuged*)

Embryo	1-day diapausing			2-day diapausing		
Egg extract	1-day diapausing	2-day diapausing	Acid treated	1-day diapausing	2-day diapausing	Acid treate
No. of cultures	32	5	23	11	3	16
Appendage formation No.	32	5	19	10	3	16
Appendage formation %		93·3			96·7	

Younger diapausing embryos. The behaviour of younger diapausing embryos *in vitro*, however, was not the same as that of the above-mentioned fully diapausing ones. Thus 1–2-day embryos developed well in the extracts from both diapausing and non-diapausing eggs of the same age as the embryos (Table 3). The culturing operation must therefore be an effective stimulus in activating the diapausing embryos which can seldom be activated *in vivo* by acid treatment. Diapausing embryos more than 2 days old became gradually less active with age in *in vitro* development (as seen in Table 4), 12-day embryos finally showing almost no growth. The final age, however, is different according to diapausing nature of explanted embryos.

In cultures made with the extract from diapausing eggs before the pigmentation of the serosa, conspicuous purplish red granules were observed on the developing embryos and amnion. It seems, from the results of experiments using many kinds

Table 4. In vitro *growth of diapausing embryos in relation to their age (five cultures each, solution 2, 1-day yolk, centrifuged)*

Age of explants (day)	Distribution of days until appendage formation					Total
	2	3	4	5	7	
1	1	4	—	—	—	5
2	2	2	—	—	—	4
4	—	1	1	1	—	3*
8	—	—	3	1	—	4
12	—	—	—	—	—	0

* Two cultures were discarded owing to bacterial infection.

Table 5. *Possible recovery in nutritive effect of the yolk in diapausing eggs kept at* 25° C. *continuously*

	Extract from eggs		
	20–40-day-old	50–90-day-old	140-day-old
No. of cultures	56	14	32
Appendage formation No.	19	4	22
Appendage formation %	33·9	28·6	68·8

1–2-day embryos were explanted.

of egg-colour mutants as material, that development of explants in media which contain 3-hydroxykynurenine is a necessary condition for the occurrence of these granules in cultures. This phenomenon recalls Horikawa's investigation on the pigmentation of eye disks of *Drosophila* in *in vitro* cultures with kynurenine-containing media (Horikawa, 1956), and will be dealt with in detail elsewhere.

Age of the yolk. The effect of the age of the yolk on culturing embryos is shown in Table 5. It seems that the further the diapausing eggs age at 25° C. the less suitable their yolk becomes for *in vitro* culture of embryos. This change in the yolk is parallel to the morphological and physiological changes in the yolk cells *in vivo* (Takami, 1953, 1954*a*). An interesting reverse change may possibly take place in the yolk after about 100 days' storage of the eggs at 25° C. The yolk recovers to a large extent the ability to nourish non-diapausing embryos *in vitro*, though little morphological change was noticed in the yolk cells at this time and the embryos in the same eggs had not yet recovered from diapause, showing neither hatch at 25° C. nor development *in vitro*. This recovery will need confirmation by further experiments.

Cold storage of eggs. The diapause of both the embryo and the yolk described above can be checked by cold storage of eggs. In Table 6, it is seen that the embryo and the yolk have been kept active for 20 days by keeping the eggs at 5° C. Cold storage for less than 20 days also gave similar results.

Table 6. *Cold storage at* 5° C. *of* 1–2*-day diapausing eggs (solution* 2, *centrifuged*)

Embryos from eggs	Extract from eggs	No. of cultures	Appendage formation
Stored at 5° C. for 20 days	Stored at 5° C. for 20 days	10	9
	Kept at 25° C. for 20 days	10	0
Kept at 25° C. for 20 days	Cold storage for 20 days	10	0
	Kept at 25° C. for 20 days	5	0

There is some reason to believe that the diapause of eggs may proceed to some extent even at 5° C., but in the present investigation, it could not be proved, probably because it is obscured by a strong activating effect of the explanting operation.

Heating of the egg. The non-centrifuged extract from the 2-day diapausing eggs which had previously been immersed in hot water at 60° C. for 5 min. was nearly the same in nutritive effect as the extract from non-heated eggs. Similar tests were also made with the extract from other heated eggs (75° C., a fews seconds) and these gave similar results, though heating of the centrifuged egg extract at 65° C. for 20 min. caused a considerable, but not complete, inhibition of the growth of explants. Further experiments, however, are desirable on this point.

DISCUSSION

The diapause of the silkworm egg is a maternally inherited characteristic and cannot, as a rule, be changed directly by the sperm entering the egg (Watanabe, 1924). It is evident that the diapause hormone is produced in the mother's body (Hasegawa, 1951, 1952; Fukuda, 1951) and affects the egg during the stay in the ovary. Therefore, the 'inhibitory substance' postulated by Watanabe (1924), if present, probably exists in the egg before embryo formation. On these grounds, it is likely that the diapause of the embryo is primarily determined by some condition of the yolk. Umeya's opinion (1937*a*, *b*, 1938, 1939, 1952) that diapausing embryos are always ready to develop, being inhibited only by some unfavourable condition of the yolk surrounding them, seems to be less plausible, because, as shown in Table 2, fully diapausing embryos could rarely be activated in *in vitro* culture with the egg extract suitable for the development of non-diapausing embryos. Younger diapausing embryos of the silkworm, or even a part of them, can be activated *in vitro* without any organic connexion (see below) with the yolk cells, in conformity to the Bucklin's result in *Melanoplus differentialis* (Bucklin, 1953). According to Bucklin small yolk-free fragments of the diapausing embryo explanted to hanging drops of Ringer's solution resumed development, indicating that the treatment exerts its effects directly on the individual tissues of the embryo, without the mediation of extra-embryonic structures or of any discrete endocrine organ. In this connexion, the possible recovery in nutritive effect of the yolk after more than 100 days' storage at 25° C. must be important, for it shows that the yolk may be

activated independently of the activation of the embryo, which still remains dormant in the same egg.

One-day-old diapausing eggs are highly sensitive to hydrochloric acid, all the eggs treated with this acid being changed to non-diapausing ones, while 3-day eggs are far less sensitive to the same treatment and fewer than 10% of the eggs can be activated (Watanabe, 1935). This change may be a manifestation of increasing dormancy, and can be explained in two ways: (1) the 'inhibitory substance' may have increased in quantity; or (2) some changes in the physiological processes concerned in diapause have progressed so far that they cannot be reversed. According to the first explanation, the termination of diapause necessitates consumption of the inhibitory substance under the effect of low temperature (Muroga, 1951).

The 'inhibitory substance', however, may not necessarily exist continuously in diapausing eggs; it may be present for a short time only, acting at a very early stage of embryonic development, and this may be sufficient for the determination of diapause. But no decisive results on this point have been obtained by the present experiments at the germ-band stage, owing to activation of the embryos by explantation alone and the possible absence of the 'inhibitory substance' at this stage. The activation of operated embryos has also been noticed by Yamazaki (1938) in wheat and by Bucklin (1953) in *Melanoplus*.

To test whether the yolk itself, in addition to the embryo, is also activated by the operation, preliminary experiments were carried out in which cultures were made with the extract from previously heated eggs. But more experiments are needed on this point. Schmidt & Williams (1953) have shown that the growth-promoting hormone contained in the active blood of the *Platysamia cecropia* silkworm could withstand heating at 75° C. for 15 min. It has recently been reported that addition of the extract from acid-treated eggs noticeably improved *in vitro* culture of the ovarian tissue in the silkworm, *Bombyx mori* (Wyatt, 1956), though whether the extract contains any hormone or not must be determined by further experiments.

The yolk of diapausing eggs is markedly different morphologically and physicochemically from that of non-diapausing eggs, so it is likely that, apart from such specific substances as the 'inhibitory substance' and the growth-promoting hormone, there is also some difference between these yolks affecting the availability of their nutritive components. This is a problem that could be made clear by means of *in vitro* culture.

The above results obtained in the silkworm are similar, in some respects, to those obtained by Yamazaki (1952) in wheat. He found that the endosperm of non-resting seed accelerates germination of the embryo transplanted into it, and that growth of the active embryo from non-resting seed cannot be inhibited by transplantation into the inactive endosperm of resting seed. He explained these phenomena by postulating the existence of some germination-promoting substance in both the embryo and endosperm of the non-resting seed, and of a germination-inhibiting substance in those of the resting seed. In contrast to this it has been proved by Schmidt & Williams (1953) that spermatogonia and spermatocytes from the diapausing pupae of *Platysamia cecropia* and *Samia walkeri* can be cultured

successfully up to the spermatid stage *in vitro* with the active blood from non-diapausing individuals. These results show that post-embryonic diapause is probably different physiologically from embryonic diapause.

The yolk of the silkworm egg is not merely a mass of non-living nutritive material, but is found in the egg as an inclusion of yolk cells. The yolk cell, or yolk spherule, is a living unit containing some yolk nuclei derived from cleavage nuclei in addition to a large quantity of yolk material, and can move independently (Takami, 1954*b*). These yolk cells keep an organic connexion with the embryo *in vivo*, it is possible therefore, that any change in the yolk will be reflected in the embryo and vice versa. In fact, detailed investigations have shown that the diapause of the silkworm egg is, strictly speaking, not a maternally inherited character, but is affected directly, to some extent, by the spermatozoon fertilizing the egg (Fujiwara & Ōyanagi, 1956; Kutsukake & Kuroiwa, 1951). In this sense, the possible recovery in nutritive effect of the yolk, independently of, or preceding, embryonic activation, is interesting in relation to Umeya's opinion that activation of the silkworm egg takes place in the yolk first, and that as a result of it the embryo becomes active secondarily. However, it has been proved by the author, contrary to his expectation, that the embryo is dormant in the diapausing egg. To make these points clear, experiments are now in progress to see whether the embryo free from its native yolk, and the yolk separated from the embryo, can be activated independently.

SUMMARY

1. Experiments have been carried out on the *in vitro* culture of silkworm embryos with silkworm egg extracts.

2. Non-diapausing embryos can be cultured well beyond the stage of appendage formation with the extract from non-diapausing eggs, while fully diapausing embryos at about the 30th day are, for the most part, unable to grow in the same extract.

3. 1–2-day-old diapausing embryos still retain the ability to develop *in vitro* as well as non-diapausing ones.

4. The extract from 1–2-day-old diapausing eggs has nearly the same nutritive effect on the *in vitro* culture of embryos as that from non-diapausing eggs, though the extract becomes less nutritive with age of eggs.

5. Diapause of both embryo and yolk can almost be prevented by cold-storage at 5° C.

6. The yolk of diapausing eggs kept continuously at 25° C. possibly recovers, after about 150 days, its nutritive effect, which falls with the onset of diapause. This change is independent of activation of the embryo, and is accompanied by little morphological change in the yolk cells.

I am very grateful to Dr T. Yokoyama, the director of the station, and to Dr H. Harizuka, the head of the department, for valuable criticisms of the manuscript. I would also like to thank Mr T. Kitazawa and Miss K. Noguchi for their assistance in the experiments.

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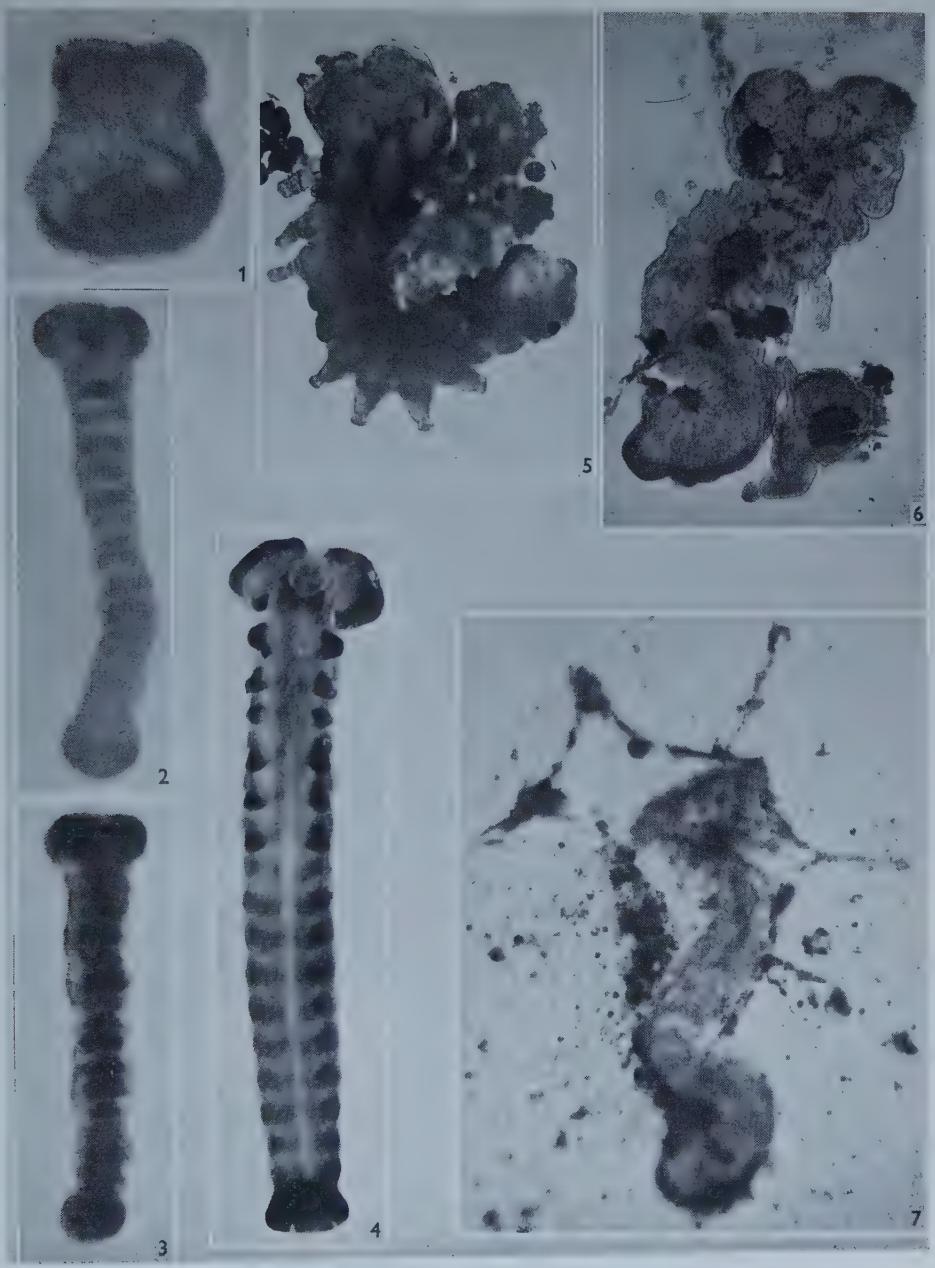
EXPLANATION OF PLATE 9

Fig. 1. A 2 hr. old germ-band.
Fig. 2. A 20-day-old diapausing embryo.
Fig. 3. A 50-day-old (fully diapausing) embryo.
Fig. 4. An embryo at the appendage formation stage.
Fig. 5. *In vitro* growth of a 2-day-old diapausing embryo (after 12-days' culture).
Fig. 6. *In vitro* growth of an acid-treated embryo with a developing embryonic fragment contained in the medium.
Fig. 7. *In vitro* growth of an embryo with spreading-out of the amnion.



TAKAMI—*IN VITRO* CULTURE OF EMBRYOS IN THE SILKWORM, *BOMBYX MORI* L. I

(*Facing p.* 296)

THE ABSORPTION OF GLUCOSE FROM THE ALIMENTARY CANAL OF THE LOCUST *SCHISTOCERCA GREGARIA* (FORSK.)

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INTRODUCTION

In a recent investigation (Treherne, 1957) it was demonstrated that glucose absorption in the cockroach was largely confined to the mid-gut caeca. It was also shown that the amount of glucose absorbed was related to crop emptying, so that the limiting process in absorption was not the transfer of glucose across the gut wall but the rate at which fluid was allowed to leave the crop. This made it impossible to study the process of glucose absorption in the caeca, which was effectively masked by the relatively slow emptying of the crop. In the present investigation, therefore, glucose absorption has been studied in the adult locust, in which it was found possible to fill the whole alimentary canal and thus to eliminate the effect of crop emptying on the total glucose absorption.

METHODS

All experiments were performed on adult female *Schistocerca gregaria* (Forsk.), reared and maintained at 28·0 ± 1·0° C., which had been isolated in glass jars and deprived of food for 24 hr.

Two methods were used to fill the alimentary canal with the experimental solutions used in this investigation. In the first, the insect was anaesthetized with CO_2 and was force-fed through a short length of 0·5 mm. bore nylon tube which was sealed into the mouth with wax. By this method the crop was filled with experimental solution and the absorption of glucose was followed as the fluid passed along the remainder of the alimentary canal. With the second method a fine nylon hypodermic needle was made by drawing out 0·5 mm. diameter nylon tube over a low flame and mounting this on an ordinary hypodermic needle holder. This nylon needle was inserted into the rectum of an anaesthetized insect and sealed into position with wax. When the insect had recovered from the anaesthetic the experimental solution was forced into the gut from an 'Agla' syringe and the needle withdrawn from the rectum. It was found that a volume of 0·15 ml. was sufficient to fill the whole of the hind- and mid-gut. The wax used was a mixture of bee's wax and resin given by Krogh & Weis Fogh (1951).

The experimental solution used in this investigation was based on the saline used by Hoyle (1953) and had the following composition:

Glucose	0·02 M/l.	$MgCl_2$	0·002 M/l.
NaCl	0·140	$KHCO_3$	0·004
$CaCl_2$	0·002	KH_2PO_4	0·006

When the concentration of the glucose was altered (i.e. to 0·002 and 0·20 M/l.), the total osmolarity of the solution was maintained by altering the NaCl concentration.

The technique used to determine the percentage absorption of glucose from the lumen of the gut was essentially similar to that described by Treherne (1957). With this technique the experimental solution contained the dye Amaranth (Azo-Rubin S) to which was added varying amounts of ^{14}C-labelled glucose. The dye, which was not absorbed from the lumen of the gut, was used as a marker and the net percentage absorption was determined by comparing the ratio of dye to glucose in the various parts of the alimentary canal. The concentration of the dye was determined in solution at pH 10·0 using a Unicam absorptiometer at an absorption maximum of 510 mμ. The fore-gut and caeca of *Schistocerca gregaria* contained a dark coloured material which tended to interfere with the colorimetric determination at the dye concentration previously used. In the present investigation, therefore, the dye concentration was raised from 0·008 to 0·05 M/l. which reduced the effects of the interfering substances. The radioactive glucose molecules were generally labelled with ^{14}C and were assayed in solution using a thin windowed G.M. tube (G.E.C. CV2139) as previously described (Treherne, 1957).

The separation and identification of ^{14}C-labelled substances from the haemolymph, gut contents and mid-gut tissue was accomplished by descending paper chromatography using Whatman no. 1 filter-paper. The samples were applied to the paper with silicone-lined micropipettes, approximately 2·0 μl. in volume. To separate the substances from the mid-gut tissue it was necessary to wash the surfaces of the gut wall free from surface-contaminating materials. The lumen was washed by inserting a steel hypodermic needle through the opening of the proventriculus and clearing the contents of the diverticula and mid-gut with a flow of distilled water. The surfaces were judged to be clean when all the dyestuff, which had been added to the experimental solution, had been washed away. The outer surface of the gut was washed free of haemolymph by quickly dipping the gut into two lots of distilled water. The tissue was then homogenized in 0·2 ml. of distilled water, centrifuged at 13,000 r.p.m. and the supernatant was applied to the base-line on the paper. The following four solvent systems were used in this investigation: ethyl acetate/acetic acid/water (Jermyn & Isherwood, 1949), methanol/formic acid/water (Bandurski & Axelrod, 1951), *n*-propanol/ammonia/water (Hanes & Isherwood, 1949) and *n*-propanol/ethyl acetate/water (Baar & Bull, 1953). Reducing substances on the chromatograms were detected by the silver nitrate method of Trevelyan, Proctor & Harrison (1950). To detect non-reducing carbohydrates the chromatograms were sprayed with 0·5% sodium-*meta*periodate in water (Evans & Dethier, 1957) before

treatment with silver nitrate. Control experiments showed that the various biological fluids caused no interference with any of the solvent systems used.

To assay the chromatograms for radioactivity the paper was cut into appropriate strips and then placed over a 1·0 cm. wide slit in a piece of Perspex beneath which was a thin-windowed G.M. tube. Counts were then made on 1·0 cm. wide portions of the paper until the whole length of the strip had been assayed.

To determine the identity of some unknown carbohydrates on the chromatograms these were eluted, subjected to acid hydrolysis and the products re-run on chromatograms. In the hydrolysis, acid was added to the solution to make 4·0N-HCl and the solution was then placed in a boiling water-bath for 20 min. Ion-exchange crystals were added, then centrifuged off and the liquid dried *in vacuo* over P_2O_5 before being re-run on a chromatogram.

RESULTS

To study the absorption of glucose from the fluid passing down the alimentary canal individuals were force-fed by mouth with 0·10 ml. volumes of an experimental solution containing 0·02M/l. glucose. The results of experiments giving the absorption after 0·25, 1·0 and 5·0 hr. are illustrated in Fig. 1. It will be seen that the bulk of the absorption occurred when the solution reached the mid-gut cacea. Some absorption of the remaining glucose occurred as the fluid passed along the rest of the alimentary canal.

The percentage glucose absorption was also investigated in experiments in which the alimentary canal was filled via the rectum with 0·15 ml. of a 0·02M/l. glucose solution. Fig. 2 shows the net percentage absorption after 5·0, 15·0 min. and 1·0 hr. The glucose disappeared most rapidly from the caeca and to a lesser extent from the ventriculus. There was no significant uptake from the hind-gut.

To study the effect of concentration on glucose uptake the net percentage absorption was measured at concentrations of 0·002, 0·02 and 0·20M/l. In these experiments the alimentary canal was filled via the rectum and all measurements were made after 0·25 hr. In Fig. 3 the absorption at 0·02M/l. is compared with that at 0·002M/l. and it will be seen that the absorption was similar at the two concentrations. When the concentration was raised to 0·20M/l. there was less percentage absorption from the caeca than at 0·02M/l. This difference was statistically significant ($P = < 0{\cdot}01$). The disappearance of glucose from the ventriculus, however, was similar at the two concentrations.

The disappearance of glucose from the lumen of the gut was followed *in vitro*. The alimentary canal was removed from an anaesthetized insect and filled via the rectum with 0·15 ml. 0·02M/l. glucose. The canal was then closed by ligatures at the crop and rectum and suspended for 15 min. in 1·0 l. of saline, to which had been added KCN and iodoacetic acid, each to a concentration of 2·0 mM/l. A stream of air bubbles ensured an adequate circulation of saline around the suspended gut. The net percentage absorption was found to be similar to that from the intact insect (Fig. 4).

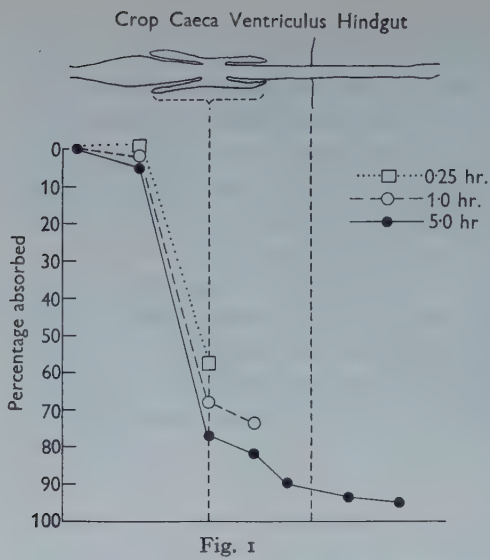

Fig. 1

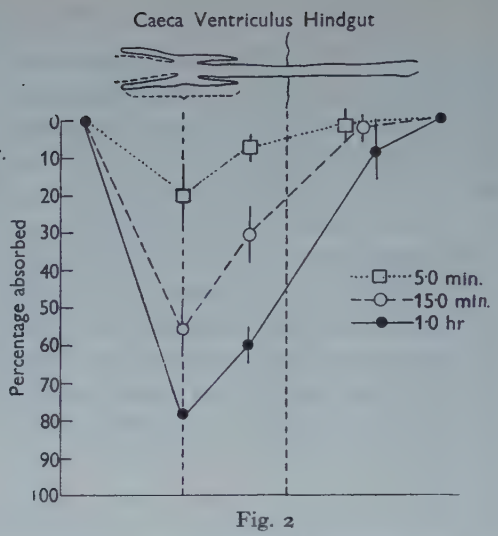

Fig. 2

Fig. 1. The net percentage absorption of glucose, from fluid passing along the alimentary canal, at three time intervals after force-feeding by mouth with 0·02 M/l. glucose solution.

Fig. 2. The net percentage absorption of glucose at varying times after the injection of a 0·02 M/l. glucose solution into the alimentary canal via the rectum. Each point is the mean of five determinations, the vertical lines represent the extent of the standard deviation.

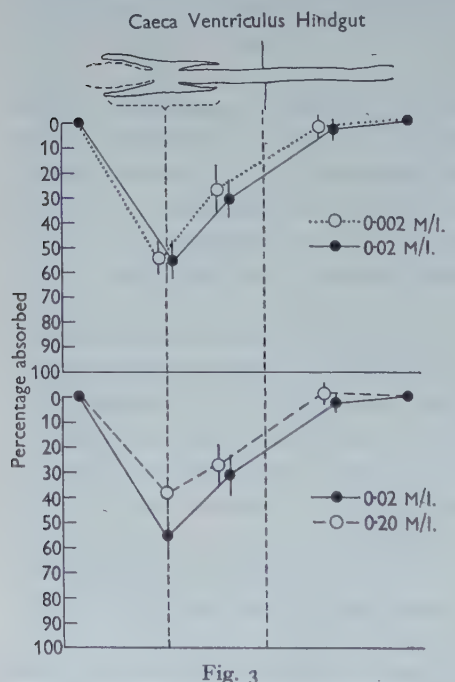

Fig. 3

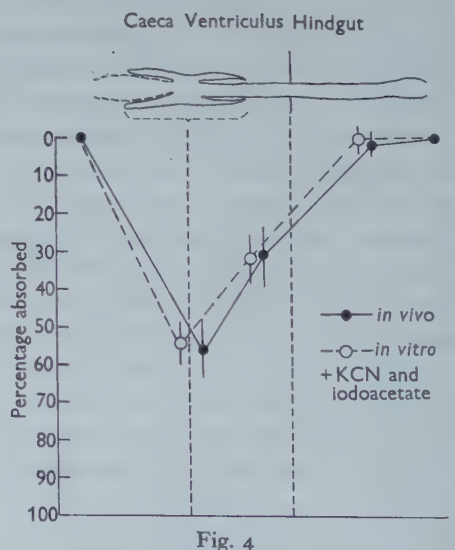

Fig. 4

Fig. 3. The effect of concentration on the net percentage absorption of glucose from the alimentary canal filled via the rectum. The symbols represent the mean and the extent of the standard deviation for five determinations.

Fig. 4. The net percentage absorption from a poisoned gut isolated in saline, compared with that from the alimentary canal of an intact insect. Each point is the mean of five determinations, the vertical lines representing the extent of the standard deviation.

The fate of the ^{14}C-labelled glucose was followed using the technique of paper chromatography. As in previous experiments 0·15 ml. vol. were injected into the alimentary canal via the rectum. Subsequently, 2·0 μl. samples of the haemolymph and gut contents were run on chromatograms, together with control spots of non-radioactive glucose, which were detected by spraying with silver. In Fig. 5 the

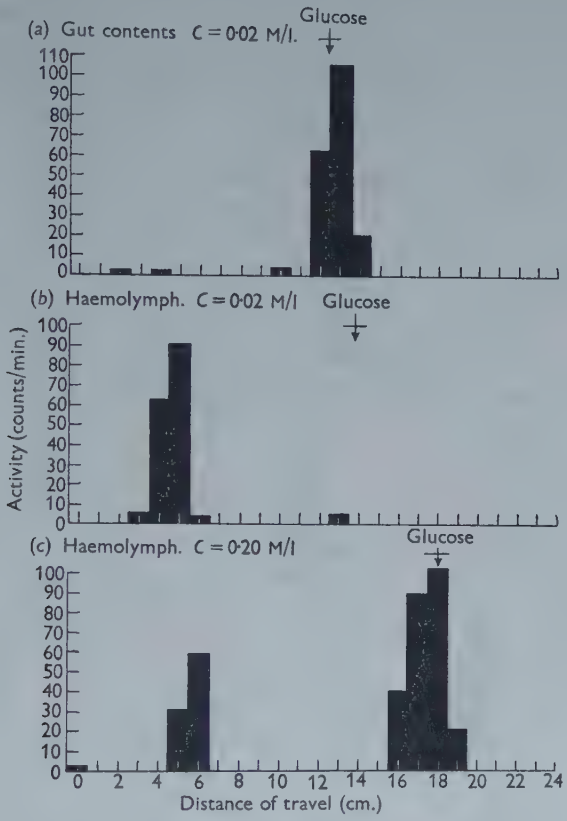

Fig. 5. Radioactivity on paper chromatograms of the caeca contents and haemolymph after injection of ^{14}C-labelled glucose into the alimentary canal. The symbols above the histograms represent the position and extent of control spots of glucose run adjacent to the experimental ones. C = glucose concentration in the gut lumen.

distribution of radioactivity in the contents of the caeca and the haemolymph is shown for chromatograms with ethyl acetate/acetic acid/water as the solvent. These results showed that the ^{14}C in the lumen of the gut was still incorporated in glucose molecules, for the radioactive peak invariably coincided with the control glucose spots. The ^{14}C recovered from the haemolymph was, however, no longer incorporated in the glucose molecule for the radioactive peak moved less than half the distance of the control glucose spot (Fig. 5*b*). This result was obtained with a glucose concentration in the experimental solution of 0·02 M/l. When the concentration in the alimentary canal was raised to 0·20 M/l. a second radioactive peak

appeared in the glucose position (Fig. 5*c*). Thus at this concentration much of the ^{14}C in the haemolymph remained as glucose.

To determine the identity of the substance causing the unknown peak of radioactivity in the haemolymph, chromatograms were run with four different solvents and control spots of possible substances were run beside the radioactive ones. Of the various substances tested the only one which consistently coincided with the radioactive peak was trehalose (α-D-glucopyranosyl α-D-glucopyranoside) (Fig. 6). This identification was also supported by the fact that the radioactive spot was strongly reducing only after treatment with 0·5 % sodium *meta*periodate, indicating the presence of a non-reducing carbohydrate.

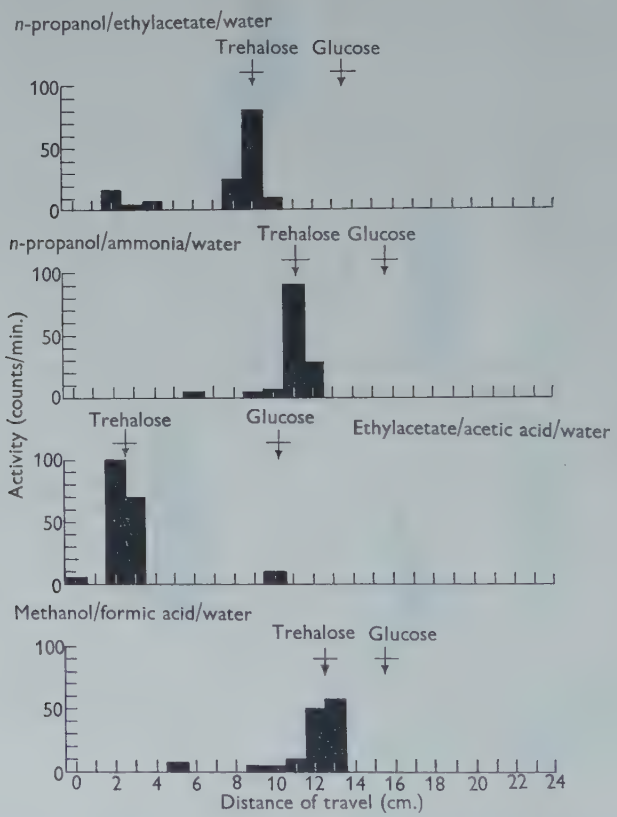

Fig. 6. The radioactivity of chromatograms of haemolymph after injection of 0·02 M/l. ^{14}C-labelled glucose into the alimentary canal. The symbols above the histograms represent the position and extent of control spots of trehalose and glucose run adjacent to the experimental ones.

^{14}C-labelled substances in the gut tissue were examined by paper chromatography. The alimentary canal was filled with a 0·02 M/l. glucose solution and after a suitable period the caeca tissues were washed and the tissue homogenate run on chromatograms. Control of trehalose and glucose were added for comparison. The results obtained were rather variable, but in most cases there was no peak of radio-

activity coinciding with the control trehalose spot. Several unknown radioactive peaks occurred in various positions on these chromatograms. Acid hydrolysis of a spot eluted from the base-line ($R_G = 0$) caused the radioactive products to appear as glucose when re-run on fresh chromatograms. This result suggests the presence of glycogen in caeca tissue.

DISCUSSION

It is evident that, as in the cockroach (Treherne, 1957), most of the glucose was absorbed from the lumen of the mid-gut caeca in the locust. The experiments in which individuals were fed by mouth showed, however, that a smaller proportion may be absorbed from the lumen of the ventriculus. A disappearance of glucose from the ventriculus was also demonstrated in the experiments in which the alimentary canal was filled via the rectum. This disappearance may not be entirely due to an uptake by the ventriculus, for it is possible that glucose may have diffused into the caeca as the concentration there fell. Thus it should be borne in mind that all of the glucose which disappeared from the lumen of the ventriculus may not have been absorbed into the haemolymph by this organ.

The chromatograms showed that the absorbed glucose had been incorporated as trehalose in the haemolymph. This non-reducing disaccharide was first reported for several species of insects by Wyatt & Kalf (1956). Its presence has since been demonstrated in the haemolymph of *Schistocerca gregaria* by Howden & Kilby (1956), and has recently been encountered in the blowfly *Phormia regina* by Evans & Dethier (1957). The present experiments showed that with an initial concentration of 0·02 M/l. all of the glucose was incorporated as trehalose, but that at 0·20 M/l. relatively large amounts remained unchanged in the haemolymph. Now the percentage absorption of glucose from the caeca was shown to be the same at concentrations of 0·002 and 0·02 M/l., but was depressed at 0·20 M/l., the level at which excess glucose accumulated in the haemolymph. These results are not inconsistent with the hypothesis that the limiting factor in the absorption of glucose is a diffusion process. Thus it would be expected that the amount of glucose leaving the lumen would be proportional to the concentration difference across the gut wall and for the net percentage absorption to be constant, as indeed it was at 0·002 and 0·02 M/l. glucose. At 0·20 M/l. the presence of appreciable amounts of glucose in the haemolymph would tend to reduce the concentration gradient so that the net percentage absorption would be reduced. The conversion of glucose would, therefore, operate to maintain a steep concentration gradient across the gut wall and thus to facilitate diffusion. The formation of trehalose involves a virtual doubling of the molecular volume which might tend to restrict back diffusion into the gut lumen.

The validity of the hypothesis outlined above was tested by comparing the disappearance of glucose from the gut of an intact insect with its disappearance from a gut isolated *in vitro*. In this experiment the isolated gut was suspended in a relatively large volume of circulating saline, so that the glucose concentration in the fluid bathing the gut was always at a very low level. Any possible effects of tissue metabolism on glucose movement were eliminated by adding iodoacetate (which is

known to depress glucose transport in mammals (Wilbrandt & Laszt, 1933)) and cyanide to the saline. This system approximates to the condition in which glucose is absorbed by diffusion through the gut wall, the steep concentration gradient being maintained by the rapid conversion of glucose to trehalose in the haemolymph. The fact that the amount of glucose absorbed in this model system was similar to that in the intact animal supports this hypothesis and suggests that the cell metabolism is not of primary importance in glucose absorption.

Howden & Kilby (1956) found that the concentration of glucose in the haemolymph of the locust was 50–100 mg %. Thus at the lowest glucose concentration used in these experiments (0·002 M/l.) the glucose would initially have been at a lower concentration in the gut lumen than in the haemolymph. Yet the percentage absorption at this concentration was similar to that at 0·02 M/l., both *in vivo* and in the isolated preparation. Thus, according to the above hypothesis, it must be assumed that even at low concentrations the conversion to trehalose is effective in maintaining an adequate local concentration gradient across some portion of the gut wall. Alternatively it can be postulated that there is a secretory mechanism which becomes effective at low concentrations, although in the absence of any evidence for such a process the former hypothesis would seem to be more acceptable.

It has often been suggested that the mechanism of hexose absorption in the mammalian intestine might be achieved simply by a conversion to some other compound, thus increasing the diffusion gradient into the mucosal cells. Such a reaction was first proposed by Höber (1899). This thesis was later developed by Verzár who suggested a possible conversion of glucose to glycogen (Verzár, 1931) or a phosphorylation of glucose in the mucosal cell (Verzár & MacDougall, 1936). More recent work on mammals has, however, not supported this hypothesis. Bárány & Sperber (1939), for example, demonstrated a true active transport of glucose against a concentration gradient, while Campbell & Davson (1948) have shown that a synthetic glucose derivative, 3-methylglucose, which is not phosphorylated *in vivo* is absorbed rapidly against a concentration gradient in the cat. Serious theoretical objections have also been raised by Wilbrandt (1954). Glucose absorption in this insect would thus seem to be fundamentally different from the process in the mammalian intestine. In fact, something like the simple facilitated diffusion mechanism originally suggested by Höber & Verzár would seem to be adequate to account for the facts observed in the absorption of glucose from the alimentary canal of *Schistocerca gregaria*.

The chromatograms of the caeca tissue showed that the ^{14}C originally present as glucose was found in a variety of compounds. These compounds were not identified, although there was good evidence that some of the ^{14}C was incorporated in glycogen. It seems most reasonable to suppose that these compounds represented various stages in the utilization of glucose by the cell's metabolism. It may thus be necessary to distinguish between that glucose which was translocated and that which was retained by the tissue for its own metabolic needs. This possibility has also been visualized in the absorption of glucose from the small intestine of mammals (Fisher & Parsons, 1950).

SUMMARY

1. The absorption of glucose from the alimentary canal of *Schistocerca gregaria* has been studied by filling the gut with a saline solution containing ^{14}C-labelled glucose together with a dye, Amaranth, which was used as a marker. The net percentage absorption was calculated from the glucose/dye ratio in the various parts of the alimentary canal.

2. The bulk of the glucose was absorbed from the mid-gut caeca, smaller amounts being absorbed by the ventriculus.

3. Glucose absorption was studied at concentrations of 0·002, 0·02 and 0·20 M/l. in solutions in which the total osmolarity was maintained by altering the NaCl concentration. The percentage absorption was similar at concentrations of 0·002 and 0·02 M/l., but was significantly less at 0·20 M/l.

4. The fate of the ^{14}C-labelled glucose was followed using paper chromatography. The glucose was shown to be rapidly converted to trehalose in the haemolymph. At a concentration of 0·20 M/l. this mechanism became saturated and excess glucose accumulated in the haemolymph.

5. The absorption of glucose *in vitro*, from a gut suspended in a relatively large volume of poisoned saline, was found to be similar to that in the intact insect.

6. From these observations it is suggested that glucose is absorbed by diffusion across the gut wall and that the process is facilitated by the rapid conversion of glucose to trehalose in the haemolymph, which tends to maintain a steep concentration gradient across the gut wall.

I am indebted to Dr F. A. Isherwood and Mr F. C. Barrett, of the Low Temperature Research Station, for helpful advice on techniques of paper chromatography.

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OXYGEN CONSUMPTION AS A FUNCTION OF SIZE AND SALINITY IN *METAPENAEUS MONOCEROS* FAB. FROM MARINE AND BRACKISH-WATER ENVIRONMENTS

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INTRODUCTION

It is common knowledge that in many euryhaline invertebrates the rate of oxygen consumption is related to the salinity of the medium. Several cases have been recorded where reduction in salinity of the medium results in increased oxygen consumption. Except for the studies of Schlieper and his associates, and the recent contribution of Lofts (1956), the problem of acclimatization of respiratory rate to lowered salinity in natural populations of a given species has not received much attention. The following is a contribution to this problem.

MATERIAL AND METHODS

The prawn, *Metapenaeus monoceros* Fab., is common along the coast of Madras, occurring in large numbers both in the sea and in brackish waters at river mouths. Prawns collected from the sea were kept in sea water in concrete aquaria in the laboratory, and those collected from the Cooum River, near Madras, were kept in sea water diluted to a salinity of 20‰. The salinity of sea water at the time of these experiments was 33·5‰ and that near the river mouth where the prawns were collected was 20‰. The Cooum River flows into the sea only during the monsoon period, and during the rest of the year the river mouth is blocked by a bar of sand. For this reason the salinity near the blocked mouth of the river varies from time to time, but is generally much lower than that of the sea during the greater part of the year (Gopalakrishnan, 1953). Further, the population of prawns within the brackish water is cut off from the sea by the sand bar during most of the year.

Oxygen consumption was estimated by the Winkler method as given in Welsh & Smith (1953). Oxygen consumption of each prawn was studied in four media, namely 100% (33·5‰), 50% (16·75‰) and 25% (8·4‰) sea water as well as in tap water. Change from one salinity to another was abrupt. Each prawn was allowed to remain for 15 min. after change into a given salinity before readings were started. All experiments were carried out at room temperature, which varied between 30 and 32° C. The prawns were not given any food once they were brought to the laboratory. After collection they were allowed to remain for at least 36 hr. in the aquaria before any of them were used for experimentation. The respiratory chamber was kept dark by covering it with black paper. Each prawn was killed immediately

after it had been studied in all the four media, and its wet weight was taken after the moisture on its body and its gill chambers had been removed with blotting paper. All the prawns used in these experiments were in the middle intermoult stage, other stages having been discarded.

RESULTS

Oxygen consumption in relation to body weight and the salinity of the medium

It is seen from Figs. 1 and 2 that the oxygen consumption per animal per hour increases with increasing size in both the groups of prawns and in all salinities. But this increase is not to the same degree in all the cases. This is evident from the *b* values given along with the graphs. But in both the cases the *b* value does not exhibit any systematic change in relation to the salinity of the medium.

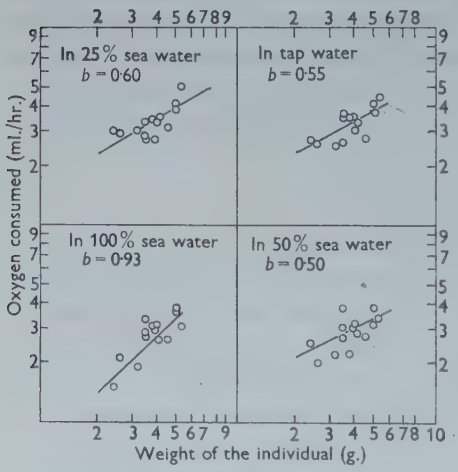

Fig. 1

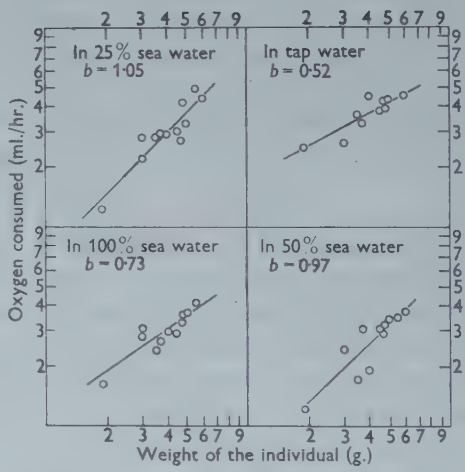

Fig. 2

Fig. 1. Oxygen consumption as function of body weight in media of different salinity, in marine *Metapenaeus monoceros*. Double logarithmic grid. Each point represents a single individual. The straight line is the best fit as per the method of least squares.

Fig. 2. Oxygen consumption as a function of body weight in media of different salinity in brackish-water *M. monoceros*. Other conditions as in the previous figure.

When marine prawns are transferred to dilutions of sea water their respiratory rate increases till it reaches a maximum value in 25% sea water, after which it falls to a lower level in tap water (Fig. 4). The brackish-water prawns behave differently. They exhibit the minimum metabolic activity in 50% sea water and their oxygen consumption increases in 100% sea water as well as in tap water and 25% sea water. It is highest in tap water and there is no indication of any adverse effect.

In Table 1 are given calculated values showing the rise in oxygen consumption with the change in salinity of the medium in individuals 3·5 g. in weight, both from the marine and from the brackish-water samples. For easy comparison, the rate in

sea water for marine prawns and that in 50% sea water for brackish-water prawns has been taken as 100 and the values for consumption in other salinities are given as percentages of these basal values. Further, the difference in the osmotic concentration between the blood of the prawn and the medium in which the oxygen consumption was measured is also given in mM/l NaCl. These values are calculated from Panikkar & Viswanathan (1948). It may be seen that in the brackish-water prawns the oxygen consumption increases with the increasing difference in osmotic concentration between the blood and the medium. This is represented graphically in Fig. 5. In the marine prawns the same is true for the oxygen consumption in 50% and 25% sea water. But the adverse effect of tap water is reflected in a drop in oxygen consumption, although the osmotic difference between the blood and the medium is greatest. Further, the lowest oxygen consumption, obtained in 100% sea water, in marine prawns is in a medium which is considerably different from the blood in its osmotic concentration.

Table 1. *Data relating to a medium-sized prawn (3·5 g. in weight) from the marine and brackish-water samples, compared to show the percentage increase in oxygen consumption in relation to the osmotic difference between the internal and external media.*

Blood osmotic concentration of marine prawns is taken as 400 mM/l NaCl and that of the brackish-water prawns as 335 mM/l NaCl. Oxygen consumption is shown as percentage increase of the rate in 100% sea water for marine prawns and in 50% sea water for brackish-water prawns.

External medium	Oxygen consumption		Osmotic difference: medium–blood	Population
	ml./g./hr.	% of basal		
100% sea water	0·67	100	+175	Marine
50% sea water	0·80	119	−115	
25% sea water	0·914	136	−260	
Tap water	0·90	134	−390	
100% sea water	0·80	121	+240	Brackish water
50% sea water	0·66	100	− 50	
25% sea water	0·74	112	−195	
Tap water	1·00	152	−335	

Marine and brackish-water prawns compared

A comparison of the oxygen consumption of the marine and the brackish-water populations is presented in Figs. 3 and 4. It is seen from this that at the two intermediate salinities, namely, 50 and 25% sea water, the marine prawns exhibit higher rates of oxygen consumption. The situation is reversed in 100% sea water, in which it is the brackish-water prawns that show a higher metabolic rate. A similar reversal is found in tap water also. Further, it may be seen from Fig. 5 that a lowering of the salinity of the medium involving the same degree of difference between the external and internal media results in a higher percentage increase in oxygen consumption in marine prawns as compared to the brackish-water prawns. Thus, for example, an osmotic difference of −115 mM/l NaCl (marine prawns in

50% sea water) results in the increase of oxygen consumption to 119% of that in sea water in the case of marine prawns, whereas a similar osmotic difference in the case of brackish-water prawns results only in an increase to 105% of the base value (read from the curve in Fig. 5).

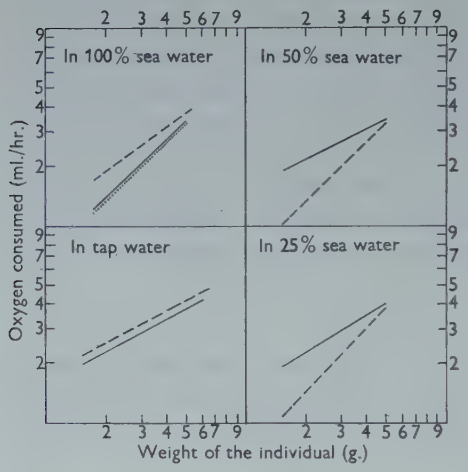

Fig. 3

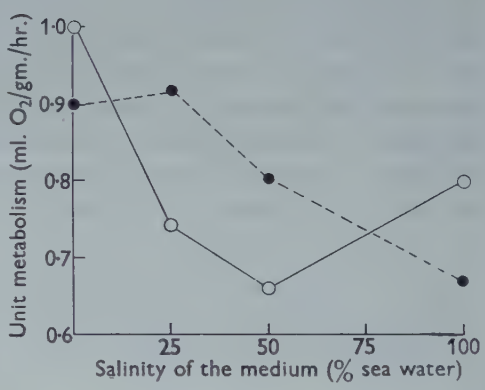

Fig. 4

Fig. 3. Comparison of oxygen consumption of marine and brackish-water prawns in media of different salinity. Curves taken from Figs. 1 and 2. Solid lines for marine prawns and broken lines for brackish-water prawns. The dotted line in upper left-hand square represents the oxygen consumption of brackish-water prawns in 50% sea water for comparison with the marine prawns in 100% sea water.

Fig. 4. Oxygen consumption as a function of the salinity of the medium in an average sized individual (3·5 g. in weight) from the marine and brackish-water samples. The values are taken from the lines given in Figs. 1 and 2. Open circles and solid line represent the brackish-water prawn and solid circles and broken line represent the marine prawn.

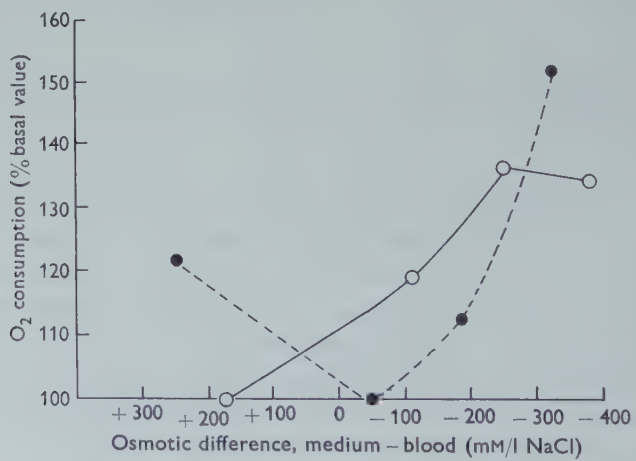

Fig. 5. Oxygen consumption (% of basal value) as function of osmotic difference between the internal and external media in an average-sized individual (3·5 g.) from the marine and brackish-water samples. Open circles and solid line represent the marine prawn and closed circles and broken ine represent the brackish-water prawn.

DISCUSSION

In general the brackish-water prawns appear to have higher *b* values. But there is no systematic trend in *b* values with change in the salinity of the medium in either population. The high *b* value reported here for brackish-water prawns in 25% sea water may have resulted from changes in physiological factors other than size *per se*. In view of these facts it is difficult to emphasize the biological significance of *b*.

The marine population of the prawn, *M. monoceros*, belongs to the second group of Bock & Schlieper (1953). It is known that *M. monoceros* exhibits active regulation of chloride (Panikkar & Viswanathan, 1948), and the increase in oxygen consumption in media of lowered salinity may be to some degree a reflexion of this regulation. But Potts (1954) has shown that in *Eriocheir* only a very small fraction of the increased oxygen consumption represents osmotic work done. Gross (1957) has suggested that a great part of the increased oxygen consumption in hypotonic media might be the result of increased locomotor activity. But it is known that in some poikilosmotic animals even tissue respiration is higher in lowered salinities (Bock & Schlieper, 1953; Schlieper, 1955), in individuals which normally inhabit media of lowered salinity. Further, Gopalakrishnan (1953) has shown that when a prawn is subjected to a constant flow of hypotonic medium while being kept in a tight glass jacket which does not permit any limb movement, its oxygen consumption increases over a period of 16 hr. and keeps steady thereafter (a result due, perhaps, to cuticular permeability). Consequently, it is not clear what the increased metabolic rates mean. But Fig. 5 is instructive inasmuch as it shows that the percentage increase in oxygen consumption is related to the osmotic difference between the blood and the medium.

In their respective natural media both the groups of prawns have about the same respiratory rate (Fig. 3). But brackish-water prawns, living in a hypotonic medium, exhibit their lowest rate of oxygen consumption in 50% sea water, while in the same medium marine prawns show an increase in their oxygen consumption which is 119% of their basal. Likewise marine prawns, naturally adapted to a hypertonic medium (100% sea water), exhibit their lowest oxygen consumption in that medium, although the gradient between the osmotic concentration of the external medium and the blood is as high as +175 mM/l NaCl, whereas brackish-water prawns in 100% sea water have a respiratory rate which is 121% of the basal. The fact that the oxygen consumption increases on transfer to a hyper- or hypotonic medium (as exhibited by brackish-water prawns in 100% sea water and marine prawns in 50% sea water), and that on prolonged sojourn in such a medium there is a marked tendency towards lowering of the oxygen consumption to its normal level (as is well exemplified by the lowered oxygen consumption of the marine prawns in 100% sea water) indicates that, besides the normal osmotic regulation resulting in an approximation of the external and internal media, a metabolic homoeostatic mechanism may be operating in relation to osmotic regulation consequent upon osmotic stress. It is possible that the long-term metabolic adjustment

may be secondary to long-term acclimatization of blood electrolytes and/or permeability (both active and passive). Whether such a homoeostatic mechanism is to be expected only amongst active regulators is an open question. But the fact that in poikilosmotic organisms, such as *Asterias rubens* (Bock & Schlieper, 1953) and *Mytilus edulis* (Schlieper, 1955) there is no such metabolic homoeostasis in media of different salinity, and thus of different osmotic pressure, is of interest in this context.

Eliassen (1952) found an increase in oxygen consumption in *Artemia salina* when the salinity of the external medium was decreased, and this was most marked in young nauplii, being less evident or even almost absent in the larger individuals. In these experiments the change to a lowered salinity was not abrupt and the larger individuals have grown to that size in media of lower salinity in which they were tested. *Artemia* takes about 4 weeks to reach maturity, and perhaps in this time there was an adaptation to lowered salinity and such an adaptation is likely to be a reason for the absence of any significant differences in oxygen consumption amongst adults in different salinities.

SUMMARY

1. The oxygen consumption in relation to the salinity of the medium has been studied in a marine and a brackish-water population of the prawn, *Metapenaeus monoceros* Fab.

2. It has been shown that the regression coefficient of oxygen consumption against weight is not the same for media of different salinity and for the two populations.

3. In both the groups of prawns an increase in the oxygen consumption was observed, with a decrease in the salinity of the medium below that of the habitat. But the marine prawns showed higher rates in 50 and 25% sea water compared to the brackish-water prawns. On the other hand, the brackish-water prawns exhibited a higher rate of oxygen consumption in 100% sea water and in tap water.

4. It is suggested that these differences might be due to (i) an osmotic adaptation, and (ii) the operation of a metabolic homoeostatic mechanism in relation to osmotic regulation.

My sincere thanks are due to the Director, Zoological Research Laboratory of the University of Madras, for giving me the necessary facilities in his laboratory for the conduct of this investigation. To Dr S. Krishnaswamy my thanks are due for several courtesies extended to me during my stay in that laboratory. I am especially grateful to Dr Theodore H. Bullock of the University of California at Los Angeles, Prof. V. B. Wigglesworth of the University of Cambridge, Dr Erik Zeuthen of the Zoophysiological Laboratory at Copenhagen, Dr W. J. Gross of the University of California at Riverside and Dr Paul Dehnel of the University of British Columbia at Vancouver for critically reading through the manuscript of this paper and offering helpful suggestions.

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SEASONAL VARIATION IN THE RESPIRATORY ACCLIMATIZATION OF THE LEECH *ERPOBDELLA TESTACEA* (SAV.)

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(*Received* 30 *October* 1957)

In a preliminary study of the oxygen consumption of leeches (Mann, 1956), it was shown that *Erpobdella testacea* has an oxygen consumption which is independent of the oxygen concentration in the water, over the range 2–6 ml./l., provided that it has been acclimatized overnight to the oxygen concentration of the experiment. The determinations in these experiments were made by enclosing the leeches in glass-stoppered bottles for periods of about 1 hr. Such experiments are unsatisfactory for a study of acclimatization because: (i) the oxygen concentration is constantly changing, (ii) the duration of the experiments is limited, and (iii) the closing of the bottles and the shaking which is necessary to ensure proper mixing of the water make it difficult to keep the animals in a quiescent state. An apparatus was therefore devised to enable the animals to be placed in a slow stream of water of any desired constant oxygen content, so that their oxygen consumption could be measured over a period of days. In this way it was possible to obtain a true resting state, and to investigate acclimatization in differing oxygen concentrations at different times of year.

APPARATUS AND METHODS

The dropping mercury electrode was used for determining the oxygen concentrations in the water. The difficulties which have to be overcome when using this method in flowing water for long periods are of two kinds. First, the speed of the water flowing past the electrodes influences the readings obtained, independently of the oxygen concentration, so it is necessary to obtain a constant, minimal flow in order to calibrate accurately. Secondly, mucus produced by the leeches tends to accumulate on the capillary and make the rate of dropping of the mercury uneven. This in turn produces erratic readings for a given oxygen concentration. The first difficulty was overcome by abandoning rubber bungs and rubber tubing as far as possible in the construction of the apparatus, and using standard glass cones and sockets for all joints, thus eliminating leaks and facilitating input and removal of experimental animals. A specially designed fine-control glass tap was used to produce a constant rate of flow (Fig. 2*b*) and the water was slowed in the vicinity of the electrodes by making the electrode chamber of relatively large dimensions. It was then found that reproducible readings could be obtained for any given oxygen concentration and rate of flow. The second difficulty, caused by the clogging

of the capillary, was overcome by adopting the wide-bore dropping mercury electrode of Briggs, Davies, Dyke & Knowles (1957) which delivers the mercury upwards at an angle of 45° through a capillary of 0·8 mm. internal diameter. The head of mercury is reduced to 15 cm. and the flow is further restricted by a middle section of capillary tubing, between the reservoir and the capillary tip, about 34 cm. long and 0·2 mm. internal diameter, bent as shown in Fig. 1.

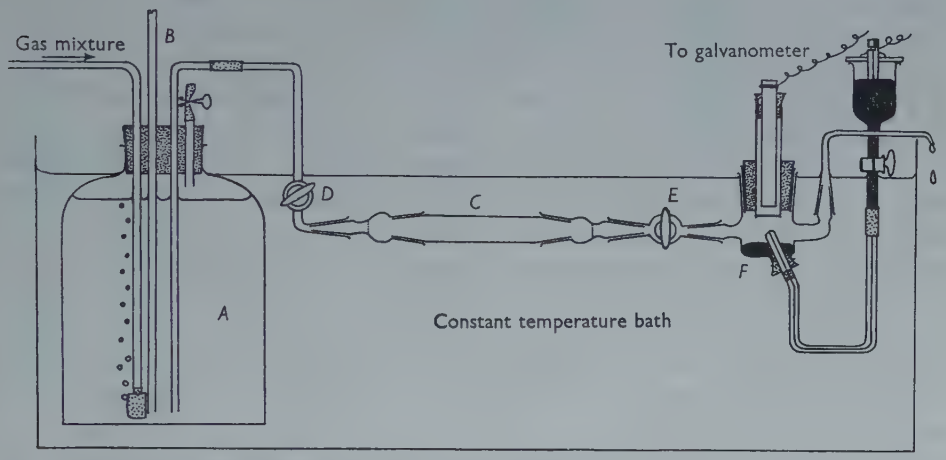
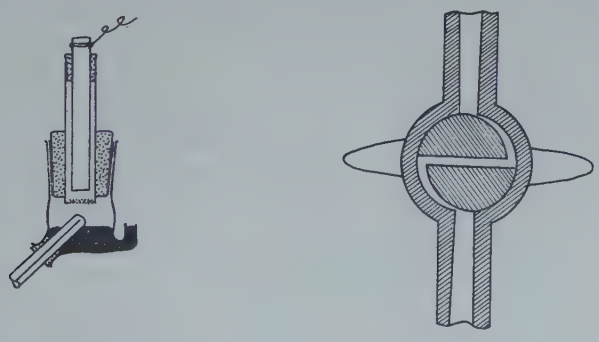


Fig. 1. Diagram showing general arrangement of the polarographic respirometer. For explanation of lettering see text. The reservoir *A* is drawn to a smaller scale than the rest of the apparatus, and is in fact a 10 l. aspirator.



a *b*

Fig. 2. (*a*) Section through the electrode chamber (*F* in Fig. 1) approximately at right angles to the plane of Fig. 1. (*b*) Section through the fine-control tap (*D* in Fig. 1) showing the tapering grooves on the key.

The general arrangement of the apparatus is also shown in Fig. 1. Water in the reservoir *A* is equilibrated with the gas mixture from a cylinder, or with air from a pump, and after equilibration the gas outlet from the reservoir is closed and a head of water built up in the tube *B*. This drives the water through the respiration chamber *C*, which has a piece of platinum gauze at each end to contain the animals. The tap *D* is the fine-control tap illustrated in Fig. 2*b*, and the effect of the fine

grooves cut in the key is to give a controlled rate of flow at any point in the range 50–500 ml./hr. The tap *E* is a standard one which may be used as a coarse control of the rate of flow, but it was also found useful because when partly closed it produced a jet of water which caused the water to be thoroughly mixed before reaching the electrode chamber. This eliminated fluctuations in the readings due to imperfect mixing of the water in the respiration chamber. The electrode chamber, *F*, is essentially a hollow glass ball with five apertures, as shown in Figs. 1 and 2*a*. The largest aperture is that at the top for receiving the zinc reference electrode. It is fitted with a 32 mm. ground glass cone and socket to facilitate opening the chamber for cleaning the capillary and the inner walls, the rubber bung being fitted into the glass cone. The other two apertures fitted with ground glass joints (10 mm.) are for the inflow and outflow of water. The tip of the capillary is led through a rubber bung. It lies in a plane at right angles to the direction of flow of water and is directed at 45° to the horizontal, with its tip in line with the inflow. The fifth aperture is for mercury overflow and is constructed so that water cannot leave by that route, even when under slightly more than atmospheric pressure. The dropping mercury electrode was kept at 0·4 V. negative with respect to the zinc electrode. The latter generates 1·0 V., so that the apparatus is working on the second step of the oxygen polarographic curve, where there is no polarographic maximum. It is not therefore necessary to add a suppressor and the apparatus can be used with any type of water. Reading tap water was used throughout the experiments, care being taken to avoid water which had passed through copper pipes as this was lethal to the leeches. Chlorine was added at a concentration of 1 p.p.m. to prevent growth of micro-organisms. The polarographic current was measured with a Cambridge Voltamoscope and the rate of flow of water was measured at the outflow using a stop-watch and a measuring cylinder.

The procedure in any experiment was first to pass the water through the apparatus with no animals present, until a steady reading was obtained over a period of several hours. Leeches were then added, and the respiration chamber wrapped in black paper. Under these conditions the leeches distributed themselves well in the chamber, and were never seen to undulate their bodies, even in water of low oxygen concentration. All experiments were carried out at $20 \pm 0{\cdot}1°$ C.

When it was required to render functionless the haemoglobin of the blood, the leeches were placed in the dark in water containing carbon monoxide at a concentration one-sixth that of the oxygen, and left until all the haemoglobin had been converted to carboxyhaemoglobin. The time required varied from 3 hr. for small leeches to 6 hr. for large ones, and in practice the latter were left in the treated water overnight in order to be ready for experiments the next day. The method used to observe the carboxyhaemoglobin was to compress a leech between a well-slide and a plain slide, in a solution of sodium hydrosulphite, and examine the preparation under a microscope with a hand spectroscope attached. The presence of two dark bands which did not fade even in these deoxygenated conditions indicated the presence of carboxyhaemoglobin. Checks of this type were made before and after each experiment.

RESULTS

A. *Oxygen consumption in relation to feeding and starvation*

In previous work (Mann, 1956) it had been shown that leeches collected from the field and subsequently starved showed a reasonably constant oxygen uptake for at least a week. Unfortunately it was not possible to obtain locally sufficient *E. testacea* for the whole series of experiments, and supplies were sent by post from the Windermere laboratory of the Freshwater Biological Association. The leeches were given a meal of *Tubifex* on arrival, and kept in well-aerated water until their oxygen uptake had reached a steady level. The pattern of oxygen consumption after feeding is shown in the first part of the curve in Fig. 3. There is a threefold increase in oxygen uptake after a meal, a decline over a period of 4 days, followed by a steady level which is maintained for several days.

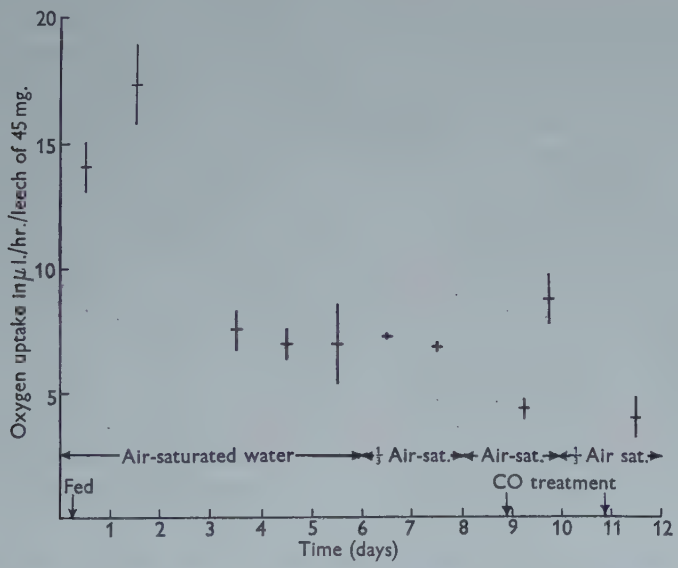


Fig. 3. Mean oxygen consumption of twenty-two *Erpobdella testacea* of average weight 45 mg. subjected to various treatments as described in the text. Lines are drawn to represent twice the standard error above and below each point.

B. *Acclimatization to low oxygen concentration in the water*

For the first series of experiments the water in the reservoir was equilibrated with a gas mixture of 11% oxygen and 89% nitrogen, so that the oxygen content was about 50% of air-saturation. Leeches, which had been fed and then starved for at least 4 days while being kept in well-aerated conditions, were placed in the apparatus and given time to settle down. In early experiments it was not always easy to obtain a steady rate of flow of water, together with a constant dropping rate for the mercury, and as readings were only taken when these conditions obtained, the number of readings which could be taken in a day was limited. In Table 1 is

Table 1. *Oxygen consumption in μl./hr. of* Erpobdella testacea *in one-half air-saturated water*

Date of start	Day 1		Day 2		Day 3		Uptake in air-saturated water	Weight (mg.)
	n	Mean and S.E.	*n*	Mean and S.E.	*n*	Mean and S.E.		
5. vi. 56	9	12·1±0·4	13	14·8±0·3**	7	14·9±0·7	15·1	92
26. vii. 56	8	15·7±2·4	11	22·7±0·9*	4	20·2±0·2	16·5	105
22. viii. 56								
Hours 1–4	—	2·1±0·4	—	—	—	—	—	—
Hours 5–7	—	8·1±1·2	8	6·9±0·2	10	8·2±0·4	9·5	55
5. ix. 56	11	6·5±0·7	12	7·2±0·6	7	8·4±0·6	9·0	50
11. ix. 56	12	5·7±0·6	9	4·8±0·4	—	—	6·5	33

** Significantly different from preceding column, $P=0.01$; * $P=0.05$.

recorded for each day of each experiment the number of readings, their mean, and the standard error of the mean. For reasons given in the previous paper, the oxygen uptake is recorded in μl. of oxygen consumed per leech of a stated weight, per hour, rather than μl./g. On the right-hand side of Table 1 is given the oxygen consumption of a leech of the same weight in air-saturated water, calculated from the results of earlier work. In the experiment of June 1956 there was a highly significant increase in oxygen consumption on the second day compared with the first, and the same result was obtained in July, except that the significance of the increase was somewhat obscured because there was a marked increase in oxygen consumption towards the end of the first day. In August the same thing happened as in July, and this has been emphasized in the table by calculating separately the mean and the standard error for the readings obtained in the first 4 hr. and those obtained later in the same day.

In the experiments of early September there is a slow increase of oxygen consumption on each of 3 days, but none of these changes is statistically significant at the 5% level of probability. By the third day the uptake is close to that obtained by enclosing the leeches in bottles containing air-saturated water, and the difference is probably accounted for by the lower level of activity in the respirometer. It seems likely, therefore, that while in the first three experiments the leeches showed an initial low level of oxygen consumption, rising as the animals became acclimatized until their uptake was comparable with that found in air-saturated water, in the fourth experiment the leeches may have been partly acclimatized before the experiment began, possibly because of exposure to water of low oxygen content before collection.

In the experiment of 11 September there was a decrease in oxygen consumption on the second day, and there is no conclusive evidence that acclimatization occurred at all. This result led to the idea that acclimatization might be more marked in early summer than at other times of the year, so another series of experiments was planned, running from winter to summer of 1957. Two changes were made in the procedure. The water was equilibrated with a gas mixture of 7% oxygen and 93% nitrogen, so that the dissolved oxygen was equal to about one-third air-saturation, and immediately before each experiment in water of low oxygen content, the oxygen

consumption of the leeches was measured in air-saturated water. The results are summarized in Table 2.

Table 2. *Oxygen consumption of* Erpobdella testacea *in one-third air-saturated water*

Date of start	Uptake in air-saturated water		*n*	Mean and S.E.	*n*	Mean and S.E.	*n*	Mean and S.E.	Weight (mg.)
	n	Mean and S.E.							
7. ii. 57	10	11·6 ± 0·9	10	4·3 ± 0·7**	4	2·2 ± 0·6	5	3·5 ± 0·01	92
9. iv. 57	4	7·2 ± 0·6	15	5·4 ± 0·2*	6	4·6 ± 0·2	—	—	40
14. v. 57	15	8·6 ± 0·4	12	7·7 ± 0·5	—	—	—	—	61
31. v. 57	9	7·0 ± 0·3	8	7·3 ± 0·01	4	6·9 ± 0·07	—	—	42
3. vi. 57	7	4·8 ± 0·4	8	2·7 ± 0·02**	13	3·6 ± 0·07**	—	—	31

** Significantly different from preceding column, $P = 0{\cdot}01$; * $P = 0{\cdot}05$.

In February the rate of oxygen consumption in oxygen-poor conditions is very low indeed, and there is no suggestion at all of acclimatization. The same is true of the April experiment, except that there is a higher level of uptake in the oxygen-poor water, even at the beginning. In May the level of oxygen uptake is approximately the same in the low oxygen concentration as in the high. Apparently we again have the condition where acclimatization may have occurred prior to the start of the experiment. It seemed possible that this might have occurred during the journey in the post, so on 3 June determinations were made with animals collected locally. Here was seen once again a low uptake on the first day, with a highly significant increase on the second, indicating progressive acclimatization.

C. *The effect of rendering functionless the haemoglobin of the blood*

After the experiments of 9–10 April when no acclimatization had occurred, and the level of oxygen consumption was $4{\cdot}6 \pm 0{\cdot}2$ μl. per leech per hour, the same leeches were placed in one-third air-saturated water containing carbon monoxide. Next morning they were transferred to the respirometer, which now contained about 0·5 ml./l. of carbon monoxide in the water to prevent dissociation of the carboxyhaemoglobin. The oxygen consumption was measured at intervals over the next 7 hr., and the mean of ten readings was $3{\cdot}6 \pm 0{\cdot}01$ μl./hr. per leech of 40 mg. This is a highly significant fall in oxygen consumption. A similar experiment was carried out after the experiment of 31 May 1957, in which the leeches had been acclimatized before the start of the experiment. The mean of eight determinations was $5{\cdot}0 \pm 0{\cdot}4$ μl./hr. per leech of 42 mg. This is again significantly lower than the value obtained for untreated leeches, and suggests that at one-third air-saturation the oxygen uptake is reduced by about 25% if the haemoglobin is rendered functionless.

A third carbon monoxide experiment was carried out in May 1957 on leeches which had been kept in well-aerated conditions for several days before the experiment. They were treated with carbon monoxide overnight and then transferred to the respirometer which was charged with water containing oxygen at one-third

air-saturation and carbon monoxide at a concentration of 0·5 ml./l. The oxygen consumption was low at first but rose steadily through the day, as shown in Table 3.

Table 3. *Oxygen consumption of* Erpobdella testacea *in one-third air-saturated water, after treatment with carbon monoxide*

Consumption before treatment	0–1·5 hr.	1·5–4·0 hr.	4·0–6·5 hr.
8·6 ± 0·4	4·1 ± 0·01**	5·5 ± 0·02*	7·5 ± 0·01*

** Significantly different from preceding column, $P = 0{\cdot}01$; * $P = 0{\cdot}05$.

After a careful check to ensure that the carboxyhaemoglobin had not dissociated it was concluded that the rise in oxygen consumption during the experiment represented an acclimatization effect, which was taking place in spite of the haemoglobin having been rendered functionless. The next step was to investigate whether acclimatization occurred in response to the oxygen shortage induced by the carbon monoxide, or whether it was a response to the low oxygen concentration in the water. Two experiments were devised. In the first, the leeches were treated with carbon monoxide overnight, and next morning their oxygen uptake was measured in air-saturated water. In the second, untreated leeches were placed in the respirometer in air-saturated water and their oxygen uptake was measured while they were receiving carbon monoxide treatment, the appropriate amount of carbon-monoxide saturated water having been added to the reservoir. The result of the first experiment was that the oxygen uptake of the leeches was the same after treatment as before. In the second, the oxygen uptake dropped sharply as shown in Table 4. This suggests that although treatment with carbon monoxide causes a fall in oxygen consumption of about 45 % in air-saturated water, leeches which have been overnight in contact with carbon monoxide have become acclimatized, and are able to take up as much oxygen when the haemoglobin is inactivated as when it is normal.

Table 4. *Consumption of* Erpobdella testacea *in air-saturated water, during treatment with carbon monoxide*

Consumption before treatment	1–2 hr.	2–3 hr.	3–4 hr.
1·8 ± 0·1	1·0 ± 0·1**	0·8 ± 0·1	1·0 ± 0·0

** Significantly different from preceding column, $P = 0{\cdot}01$; * $P = 0{\cdot}05$.

D. *A composite experiment*

To demonstrate each of the effects described in the preceding three sections, twenty-four leeches received in a batch from Windermere were given a meal of *Tubifex*, after which their oxygen consumption was followed until a steady state had been reached. They were then transferred from air-saturated to one-third air-saturated water and their uptake followed for 2 days. Next, after a period in air-saturated water, they were treated with carbon monoxide overnight and their

uptake measured in air-saturated water. Finally, the carbon monoxide experiment was repeated in one-third air-saturated water. The results are shown graphically in Fig. 3. There is no significant difference between the figures of the 4th, 5th, and 6th days, so 7 μl./hr. may be taken as the normal consumption in air-saturated water. There is no significant change when the animals are transferred to one-third air-saturated water, presumably because the animals are already acclimatized. When transferred to air-saturated water and then treated with carbon monoxide, the level of oxygen consumption fell sharply at first, but rose rapidly after 2 hr. to a level significantly higher than normal. On the last day of the experiment, when the leeches were in low oxygen concentration and had been treated with carbon monoxide, the uptake was at a low level throughout the day.

DISCUSSION

In the earlier paper on leech respiration (Mann, 1956) it was shown that *Erpobdella testacea* may have an oxygen consumption which is independent of, or dependent on, the oxygen concentration of the water, according to whether or not there has been an opportunity for acclimatization. The experiments on which this finding was based were carried out in September. It is now shown that whereas at certain times of the year this leech may become independent after acclimatization, at other times of year it does not do so. If this state of affairs is at all widespread among aquatic invertebrates it will be necessary to accept with caution the distinction which is often made (Zeuthen, 1955; Bishop, 1950) between animals having a dependent type of respiration and those having the independent type. Walshe-Maetz (1953) showed that when *Chironomus plumosus plumosus* L. is acclimatized overnight to the oxygen concentration of the subsequent experiment, its metabolism at oxygen concentrations greater than 45% air-saturation changes from dependent to independent. Hyman (1929) showed that the degree of independence shown by *Planaria* depends on the amount of acclimatization allowed, and similar results were obtained with various species of arthropod by Hiestand (1931). Acclimatization to low oxygen concentrations has been demonstrated in young speckled trout by Shepard (1955).

Walshe-Maetz was also able to show that for *Chironomus* adaptation to lower oxygen concentrations consists of an increase in the amount of respiratory irrigation in the larval tube, but van Weel, Randall, and Takata (quoted in Zeuthen, 1955) found that the Hawaiian crustacean, *Podophthalmus vigil*, was able to regulate its metabolism in conditions of falling oxygen tension without any corresponding change in ventilatory activity. Shepard (1955) thought that the acclimatization of the speckled trout was due to a change in the oxygen capacity of the blood. In considering what might be the mechanism of acclimatization in *Erpobdella testacea*, one must take into account the fact that no ventilatory activity was seen when the leeches were in the respirometer, although in the earlier experiments in which the leeches were enclosed in bottles (Mann, 1956) undulations of the body were frequently observed. A possible explanation is that in still water a leech builds up

an oxygen gradient round its body, and undulation results in a better oxygen supply, but that in the flowing water of the respirometer the oxygen gradient is broken down and no benefit is derived from body undulation, which is therefore discontinued. Whatever the explanation, the fact remains that the acclimatization observed in the present series of experiments was not brought about by undulatory activity but by some internal mechanism.

In considering the probable nature of this mechanism it is necessary to assess the importance of the haemoglobin. The experiments in this section can only be regarded as exploratory, but results to date indicate that when there is no acclimatization carbon monoxide reduces oxygen uptake by about 25% in one-third air-saturated water, and by about 45% in air-saturated water, so we may conclude that between one-quarter and one-half of the oxygen carried by the blood is carried by the haemoglobin. This is comparable with the results obtained by Johnson (1942) for the earthworm. She found that the haemoglobin of the blood was responsible for supplying 22–40% of the respired oxygen, according to the oxygen pressure of the atmosphere. At times of year when acclimatization to low oxygen concentrations in the water is occurring there is also evidence of acclimatization to carbon monoxide treatment. In the experiment of Table 4, when the leeches were in air-saturated water at all times, treatment with carbon monoxide was followed by a drop in oxygen consumption; but if the animals were left in carbon monoxide overnight their consumption by morning had returned to the same level as before treatment. Again, in the experiment of section D, treatment with carbon monoxide resulted in an initial fall in oxygen consumption, followed by a sharp rise. The experiments in which the leeches were subjected to both carbon monoxide and a low oxygen concentration in the water suggested that while the leeches might at times show signs of acclimatization, they were never able to compensate for the two factors, so that oxygen uptake after treatment never reached the same level as before treatment.

We thus arrive at the tentative conclusion that there is a mechanism of acclimatization which operates in summer but not in winter, and is independent of the haemoglobin of the blood, in the sense that acclimatization can occur when the haemoglobin is inactivated. Apparently the effect of oxygen want produced by placing the animals in water of low oxygen content is comparable with that produced by knocking out the haemoglobin, and both produce an acclimatization response at certain times of year. Perhaps the simplest mechanism which could be envisaged in the light of the known facts is that there is an increase in rate of circulation of the blood in oxygen-poor conditions. This may be combined with body undulations in still water but not in running water. Information on this mechanism is clearly required, but will be difficult to obtain, owing to the deep pigmentation of the tissues.

Finally one may consider the possible significance of this mechanism in the life of the animals. *Erpobdella testacea* has been found by the author in greatest numbers in this country in reed swamps made up of dense growths of *Sparganium* in highly organic mud. In summer the leeches may be collected from the leaf bases, where cocoons are deposited, but in winter they move down into the mud. In both

kinds of situation it is to be expected that low oxygen concentrations will occur. In winter there is apparently no attempt to regulate the metabolism, which must sink to a very low level, but in the spring and summer, when there is active growth and breeding, the leech is able to regulate its metabolism at a level comparable with that found in air-saturated water.

SUMMARY

1. In summer the leech *Erpobdella testacea* becomes acclimatized to a low concentration of oxygen in the water, and can maintain a constant rate of oxygen consumption down to one-third air-saturation. This acclimatization does not occur in winter.

2. The mechanism of acclimatization is such that it can operate when the leeches are resting, and when the haemoglobin has been prevented from functioning by treatment with carbon monoxide.

3. In the course of normal respiration in air-saturated water at 20° C. about 45% of the oxygen is transported to the tissues by the haemoglobin. In one-third air-saturated water about 25% is transported in this way.

4. After a meal of *Tubifex* the oxygen consumption increases threefold, and declines to the previous level over a period of 4 days.

5. A polarographic respirometer embodying a wide-bore dropping mercury electrode has been developed for this work. It provides on a galvanometer a constant indication of the oxygen concentration in the water which has passed over the animals.

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THE INFLUENCE OF PREOPERATIONAL TRAINING ON THE PERFORMANCE OF OCTOPUSES FOLLOWING VERTICAL LOBE REMOVAL

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INTRODUCTION

Removal of the vertical lobe from the brain produces no obvious deficiencies in the behaviour of octopuses in aquaria until attempt is made to train them to make visual or tactile discriminations (Boycott & Young, 1950; Wells & Wells, 1957*a*). It is then found that animals operated in this way learn less readily than controls; more trials are required to train them to recognize objects seen or touched and the proportion of errors made, even after prolonged training, is rarely as low as that made by controls. The difference between controls and operated animals is most marked in the case of difficult discriminations, and when training trials are relatively far apart (Boycott & Young, 1955, 1957; Wells & Wells, 1957*a*), so that the performance of animals with the vertical lobe removed most closely resembles that of controls when they are trained to make easy discriminations by means of trials at short intervals. Partial removal of the vertical lobe produces deficiencies dependent upon the proportion of this tissue removed (Boycott & Young, 1955, 1957; Wells & Wells, 1957*a*). These findings indicate that the function of the vertical lobe is, in general, similar in visual and tactile learning.

It should be borne in mind, however, that most experiments on postoperational visual learning have been made with animals trained before operation, whereas postoperational tactile learning has been studied exclusively with animals that were untrained when operated. Since no experiments had been made to determine the effect of vertical lobe removal on the performance of trained animals in tactile discriminations, the justification for comparing visual and tactile experiments was questionable. The present account is of experiments made to rectify this position and deals with the postoperational performance of animals trained to make tactile discriminations before removal of their vertical lobes. By comparing the performance of such animals with that of animals trained after operation without pretraining various conclusions can be drawn about the function of the vertical lobe in tactile learning.

MATERIAL

Octopus vulgaris Lamarck of between 300 and 800 g. from the Bay of Naples were used in this work. The animals were caught and kept in individual tanks as described by Boycott (1954) and fed upon crabs or pieces of fish, those showing signs of

damage due to rough handling at capture or failing to feed regularly being discarded.

Animals were blinded by section of the optic nerves (as described in Wells & Wells, 1956) before use in training experiments. Most of them were subjected to a further operation in which parts of the vertical and superior frontal lobes were removed either before training, or in the course of training experiments as described below; these operations to the central part of the brain were carried out as described in Wells & Wells (1957*a*).

In order to ensure complete vertical lobe removals, the lesions were deliberately made large, particularly at the anterior end of the vertical lobe where a small portion of it runs under the superior frontal lobe. Inevitably the superior frontal lobe was partially damaged in many of the animals, and in some cases the whole of this lobe was removed to ensure complete vertical lobe removal. After damage to the lateral parts of the superior frontal lobe, octopuses may fail to attack objects seen at a distance in visual experiments (Boycott & Young, 1955), but there is apparently no effect on the performance of animals in tactile training; indeed, as will be shown in a later account, removal of the superior frontal, vertical and at least a large part of the basal lobes produces animals whose performance in tactile training experiments cannot be distinguished from that of animals lacking only the vertical lobes. As a result, it has been possible to ensure that almost every 'animal lacking the vertical lobe' used in the present series of tactile experiments had the vertical lobe entirely removed, a fact that must be borne in mind when comparing these experiments with visual discrimination experiments (Boycott & Young, 1956, 1957) in which nearly all the animals used had incomplete lesions as a consequence of the operator's anxiety to avoid damage to the lateral parts of the superior frontal lobe.

Animals were killed after use in one or more experiments, and the extent of brain lesions estimated from serial sections prepared according to a modification of Cajal's method given in Sereni & Young (1932).

In the text individual animals are referred to by the number with which they were identified in our original protocols, e.g. C28NVB. The prefix C shows that the experiments were made in 1956 and D, 1957. The suffixes B, NV and NSF denote 'blind' (=optic nerves cut), 'no vertical lobe' and 'no superior frontal lobe' respectively. The categories 'no vertical lobe' and 'no superior frontal lobe' include animals having considerable areas of these parts still intact as well as those in which the lesion was complete.

METHOD

Animals were trained as described in Wells & Wells (1956). Briefly, the training consisted of a series of trials at each of which one or other of a pair of Perspex objects was presented, the animal being rewarded with a piece of fish for passing the 'positive' object to the mouth and given a small (6–9 V. a.c.) electric shock if it did the same to the 'negative' object. The animals were given no pretraining experience of the objects. In the present series animals were required to learn to discriminate between a smooth Perspex cylinder, P4, $2{\cdot}5 \times 3{\cdot}0$ cm. long and a

similar cylinder with grooves cut into it, either P1 or P8. P1 had 1 mm. deep grooves cut into it longitudinally at intervals of 3 mm., P8 had similar grooves circumferentially at 2 mm. intervals. When required to discriminate between P1 and P4, controls made about twice as many errors as in P8/P4 discriminations (Wells & Wells, 1957*a*).

Trials were arranged in a systematized order, the interval between trials being either 1 hr. ('long-term' experiments) or 5 min. ('short-term' experiments). Under the long-term conditions there were 8 trials a day (4+, 4−) arranged + − + − + + − − first day, − + − + + − − + second day and so on; the short-term trials were arranged in groups of 20 trials (10+, 10−), always in the order + − + − + + − − + − + − − + + − + − + −, there being two such groups per day, the second group beginning not less than 6 hr. after the start of the first. In both cases there was an interval of about 14 hr. between the last trial on one day and the first on the next. In the retention tests trials were arranged as in the short-term experiments but no punishments or rewards given.

EXPERIMENTAL RESULTS

(1) *Train–operate–train experiments made under the long-term conditions*

For these, animals were trained at a rate of 8 trials per day for 6 or 12 days (48 or 96 trials), operated upon at the end of the last day of training, allowed 1 day to recover, and first tested for effects of vertical lobe removal 36 hr. after the operation. Preoperational breaks in training of this length cause little or no deterioration in performance (Table 1).

Six animals were treated in this way, three being trained for 48 and three for 96 trials before operation. All had lesions including complete removal of their vertical lobes. At the end of preoperational training the performance of these animals was nearly perfect, only three errors being made by six animals in the last 2 days (=a total of 96 trials) of their training (Table 1). It is convenient for purposes of comparison with others to express this result in terms of an 'index of differential response' used by Boycott & Young for comparing the performance of animals in visual experiments. This index ('*I*') has the advantage of making it possible to compare the performance of animals whether they err predominantly by accepting the negative objects (as here) or by rejection of the positives, and is calculated by dividing the difference in number of acceptances of the positive and negative objects by the total number of trials with each. $I = 0{\cdot}0$ indicates random errors, $I = 1{\cdot}0$ perfect performance. Over the last 2 days (=96 trials; 48+, 48−) of their preoperational training, the value of I for the six animals given in Table 1 was $(48-3)/48 = 0{\cdot}94$.

When training was continued after operation it was found that the same animals at first took all the objects, both positive and negative, as at the start of their preoperational training, so that for the 2 days (96 trials) immediately after operation $I = 0{\cdot}04$. Although these animals subsequently relearned to discriminate between the objects they were slower to do this than in their original preoperational

Table 1. *Train–operate–train experiments*

(Eight trials (4+, 4−) per day. Discrimination between P4 and P8. The results with each animal are arranged in three columns; under + and − are given the number of times that the positive and negative objects were taken on each day. Column E gives the total daily errors.)

	Trained for 48 trials before operation									Trained for 96 trials before operation								
	C68 NVB NSF			C69 NVB NSF			C70 NVB NSF			C26 NVB NSF			C27 NVB NSF			C28 NVB NSF		
Proportion of vertical lobe removed	100%			100%			100%			100%			100%			100%		
	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E
	4	4	4	4	1	1	4	4	4	4	2	2	4	3	3	4	2	2
	3	3	4	4	2	2	3	2	3	3	1	2	4	2	2	3	1	2
	4	0	0	4	0	0	4	2	2	4	0	0	4	0	0	4	1	1
	4	1	1	4	0	0	4	1	1	4	0	0	4	0	0	4	0	0
	4	0	0	4	0	0	4	1	1	4	1	1	4	0	0	4	0	0
	4	0	0	4	1	1	4	0	0	4	1	1	4	1	1	4	1	1
	Operation, vertical lobe removed. 36 hr. break									36 hr. break in training								
										4	1	1	4	0	0	4	0	0
	4	4	4	4	4	4	4	4	4	4	3	3	4	0	0	4	2	2
	4	4	4	4	4	4	4	4	4	4	1	1	4	0	0	4	0	0
	4	4	4	4	4	4	4	4	4	4	1	1	4	0	0	4	0	0
	4	3	3	4	2	2	4	3	3	4	0	0	4	0	0	4	1	1
	4	1	1	4	3	3	4	4	4	4	0	0	4	0	0	4	0	0
	4	1	1	3	4	5	4	4	4	Operation, vertical lobe removed. 36 hr. break								
	36 hr. break in training																	
	4	4	4	3	2	3	4	1	1	4	4	4	4	2	2	4	4	4
	4	0	0	2	1	3	4	2	2	4	4	4	4	4	4	4	4	4
	4	1	1	1	0	3	4	3	3	4	4	4	4	1	1	4	2	2
	4	1	1	1	1	4	4	0	0	4	4	4	4	0	0	4	3	3
	4	2	2	3	2	3	4	2	2	4	3	3	4	0	0	4	2	2
	4	1	1	3	1	2	4	2	2	4	2	2	4	0	0	4	2	2
	36 hr. break in training																	
	4	0	0	3	1	2	4	3	3	4	2	2	4	0	0	4	2	2
	4	3	3	4	0	0	4	2	2	4	1	1	4	0	0	4	2	2
	4	3	3	3	0	1	4	1	1	4	1	1	4	0	0	4	1	1
	4	1	1	3	0	1	4	0	0	4	1	1	4	0	0	4	0	0
	4	1	1	3	0	1	3	0	1	4	2	2	4	0	0	4	0	0
	.	.	.	.	.	.	.	.	.	4	0	0	4	0	0	4	0	0
Index of differential response for the whole period of training																		
1. Before operation	0·63			0·83			0·54			0·75			0·88			0·81		
2. After operation	0·50			0·34			0·41			0·44			0·85			0·54		
Index for the first 2 weeks postoperational training																		
	0·46			0·15			0·29			0·44			0·85			0·54		

training (Table 1), a result which suggests that very little trace of the preoperational discrimination training survives vertical lobe removal. It is only when the performance of such animals is compared with that of others trained after operation without pretraining, that traces of preoperational learning can be seen to survive vertical lobe removal. The index of differential response for the first 2 weeks of postoperational training of the six pretrained animals shown in Table 1 was 0·46;

Table 2. *Train–operate–train experiments*

(Training at 40 trials per day in two groups of 20 (10+, 10−); for retention tests with these animals see Wells & Wells (1958). Discrimination between P1 and P4. Conventions as Table 1.)

	C173 NVB NSF			C188 NVB NSF			C189 NVB		
Proportion of vertical lobe removed	100%			100%			70%		
	+	−	E	+	−	E	+	−	E
	9	9	10	9	6	7	10	6	6
	10	5	5	10	6	6	9	3	4
	10	2	2	10	3	3	10	3	3
	9	0	1	10	4	4	10	0	0
	9	0	1	10	2	2	9	0	1
	10	1	1	10	3	3	10	1	1
	Operation, vertical lobe removed. 36 hr. break in training								
	10	1	1	10	2	2	10	4	4
	7	0	3	10	1	1	7	1	4
	.	.	.	10	5	5	10	3	3
	.	.	.	10	2	2	7	3	6
	.	.	.	10	3	3	10	1	1
	.	.	.	10	2	2	9	1	2
	.	.	.	.	.	.	7	3	6
	.	.	.	.	.	.	7	2	5
Index of differential response for the whole period of training									
1. Before operation	0·67			0·58			0·75		
2. After operation	0·80			0·75			0·61		

the index for the same period for seven animals without pretraining was 0·39 (details of the performance of these seven animals, all of which—save one with 95% removed—had the vertical lobe entirely removed, are given in Wells & Wells, 1957*a*). The difference between pretrained and non-pretrained animals was more marked in the case of those pretrained for 96 than for 48 trials; for the 2 weeks after operation I (mean) = 0·60 for three animals each trained for 96 trials before operation, and I (mean) = 0·31 for three animals trained for 48 trials.

(2) *Train–operate–train experiments made under the short-term conditions*

Results rather different from the above are obtained when animals are pretrained at a rate of 40 instead of 8 trials per day. Table 2 shows the performance of three animals each of which was trained for 120 trials before removal of its vertical lobe during a 36 hr. break in training. On the last day before operation these three animals made nine errors in a total of 120 trials (I = 0·85), and in a similar period immediately after removal of their vertical lobes fifteen errors (I = 0·75). The decline in performance produced by the operation (I = 0·85 to I = 0·75) is obviously not of the same order as that produced in comparable experiments made under the long-term conditions (where the index fell from 0·94 before operation to 0·04 afterwards), although the number of pretraining trials per animal was not dissimilar in the two cases (120 trials under the short-term conditions compared with up to 96 trials under the long). It should be noted that the animals were trained to discriminate between

P 1 and P 4 in the short and P 8 and P 4 (an easier discrimination, see p. 326) in the long-term experiments. Removal of the vertical lobe is known to have a relatively greater effect upon the performance of animals in the more difficult P1/P4 discrimination (Wells & Wells, 1957*a*), so that had the objects been alike in the two sets of experiments, the difference produced in the results would have been even more marked.

(3) *Train–operate–retain experiments*

In the long-term experiments described in §1 above, little trace of pretraining was detectable immediately after removal of the vertical lobe, while the short-term experiments reported in the last section show that under somewhat different conditions (trials five times as frequent) considerable traces of pretraining survive the operation. The long-term experiments alone might lead one to suppose that learning normally takes place almost entirely within the vertical lobe and only elsewhere if this is not present. The short-term experiments clearly show that the structural and organizational changes occurring as a result of training are not limited to the vertical lobe, and, indeed, that its removal causes very little immediate loss of memory under these conditions.

One possible explanation of this would be that the training conditions dictate where in the nervous system learning takes place, and that for some reason training at a rate of 40 trials per day produces changes that are either more widespread or limited to a different part of the nervous system than training at a rate of 8 trials per day. Another possibility is that learning takes place in the same part of the brain in the two cases, but that the long-term conditions do not reveal memories that can be shown to be present under the short-term conditions. To check this, animals were trained under the long and short conditions, operated as before, and, after the usual 36 hr. gap, subjected to identical retention tests. These were carried out in the same order as under the short-term training conditions but without reward or punishment.

The results of retention tests are given in Tables 3 and 4. Control animals were subjected to dummy operations in which they were anaesthetized and the brain exposed as usual, but no lesion made. Table 3 gives the results of retention tests made with six animals (four having vertical lobe lesions and two controls) after training to distinguish P 8 from P4 under the long-term conditions. During the last 16 trials (2 days) of their preoperational training $I = 0{\cdot}88$ for these six animals. In postoperational retention tests of 20 trials made 36 hr. later, the performance of one control was perfect ($I = 1{\cdot}0$) and the other nearly so ($I = 0{\cdot}90$). The four operated animals made a considerably higher proportion of errors, mainly by acceptance of negative objects ($I = 0{\cdot}60$, $0{\cdot}10$, $0{\cdot}30$, $0{\cdot}40$—mean $0{\cdot}35$). The performance of these animals, although poorer than that of the controls, clearly shows some retention of the preoperational training.

Table 4 gives results of a similar series of tests carried out after short-term training to discriminate between P1 and P4 or between P8 and P4. The length of pretraining in these experiments was somewhat variable as the animals were trained not for a given number of trials, but to a prescribed standard of accuracy

Table 3. *Train–operate–retain experiments*

(Pretraining at 8 trials (4+, 4−) per day, retention tests of 20 trials (10+, 10−) at 5 min. intervals without shocks or rewards. Discrimination between P8 and P4. Conventions as Table 1.)

	C132B			D9B			D1NVB			D2NVB NSF			D4NVB			D8NVB		
Proportion of vertical lobe removed	.			.			80%			100%*			90%			100%		
	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E
	4	4	4	3	3	4	4	4	4	4	2	2	2	3	5	2	1	3
	4	2	2	4	1	1	4	4	4	3	1	2	3	1	2	3	0	1
	4	1	1	4	1	1	4	2	2	4	0	0	4	2	2	3	0	1
	4	2	2	4	1	1	4	2	2	4	0	0	4	0	0	4	0	0
	4	0	0	4	2	2	4	1	1	4	0	0	4	0	0	4	0	0
	4	1	1	4	0	0	4	1	1	4	0	0	4	2	2	4	0	0
				Dummy op.			Operation, vertical lobe removed.											
	36 hr. break in training																	
	4	1	1	Retention tests														
	4	0	0	10	0	0	10	6	6	10	9	9	10	3	3	8	2	4
	4	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4	2	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Dummy op. 36 hr.			.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Retention																	
	9	0	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

* Since the entire superior frontal lobe was removed, disconnecting the vertical lobe from its tactile input (Boycott & Young, 1955) D2NVBNSF may be regarded for purposes of comparison as having the vertical lobe entirely removed, although a part of the extreme right-hand gyrus (about 15% of the total volume of the lobe) remained after operation.

before operation.* Each animal was trained under the short-term conditions until it reached a criterion of 85% correct responses in a group of 20 trials. It was then overtrained for as many trials as it had taken to reach this standard and then operated. Retention tests were carried out as before, 36 hr. after the end of training. In these tests two controls trained to discriminate between P1 and P4 made 1 and 2 errors respectively ($I = 0{\cdot}90$ and $0{\cdot}80$); five animals without their vertical lobes made between two and eight errors each (average 5·4 errors, $I = 0{\cdot}46$) and two trained to distinguish between the easier P8 and P4 made five and eight errors ($I = 0{\cdot}50$ and $0{\cdot}20$).

DISCUSSION

The effect of preoperational training on the performance of octopuses when training is continued after complete removal of their vertical lobes is summarized in Fig. 1. This shows that:

(1) Removal of the vertical lobes from animals pretrained under the long-term conditions leads to an immediate decline in performance. When training is

* With one exception, C132NVB, that had already been trained as a control in the long-term experiments.

Table 4. *Train–operate–train under the short-term conditions*

(Conventions as Table 1.)

Trained on P1/P4																						Trained on P8/P4					
	C158B*			C171B			C149 NVB NSF			C151 NVB			C154 NVB NSF			C155 NVB NSF			C158 NVB* NSF			C132 NVB† NSF			C202 NVB NSF		
Proportion of vertical lobe removed	.			.			95%			100%			100%			100%			100%			95%			90%		
	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E
	10	9	9	6	6	10	10	10	10	8	8	10	10	9	9	8	1	3	10	3	3	10	0	0	5	5	10
	10	4	4	9	3	4	10	6	6	5	0	5	10	6	6	9	2	3	10	4	4	10	0	0	2	1	9
	10	2	2	10	3	3	8	0	2	9	1	2	10	5	5	Vert. out.			10	0	0	10	0	0	7	4	7
	10	0	0	9	1	2	10	1	1	7	3	6	10	2	2										10	4	4
	10	1	1	4	1	7	8	0	2	6	0	4	10	2	2	36 hr. break			Vertical lobe removed						10	2	2
	10	0	0	6	1	5	6	0	4	5	0	5	10	2	2				36 hr. break in training						1	0	9
	Dummy operation						Vertical lobe removed						10	2	2	3	0	7							10	0	0
	36 hr. break in training															.	.	.	10	4	4	7	2	5	10	1	1
	10	1	1	10	2	2	3	1	8	10	2	2	Vert. out.			.	.	.	.	.	.	.	.	.	10	2	2
													36 hr. break			.	.	.	.	.	.	.	.	.			
	.	.	.	.	.	.	.	.	.	.	.	.	10	6	6	.	.	.	.	.	.	.	.	.	Vert. out.			
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	36 hr. break		
																									3	1	8

* Same animal. Training in right-hand column immediately follows last retention test on left-hand column.
† This animal had previous training as shown in Table 3.

continued, however, the accuracy of response approaches that attained preoperationally.

(2) Under these conditions animals that had not been trained before operation required more trials to reach a given standard of accuracy of response postoperationally than pretrained animals.

(3) Removal of the vertical lobes from animals after pretraining under the short-term conditions causes little or no disturbance to the postoperational performance of these animals.

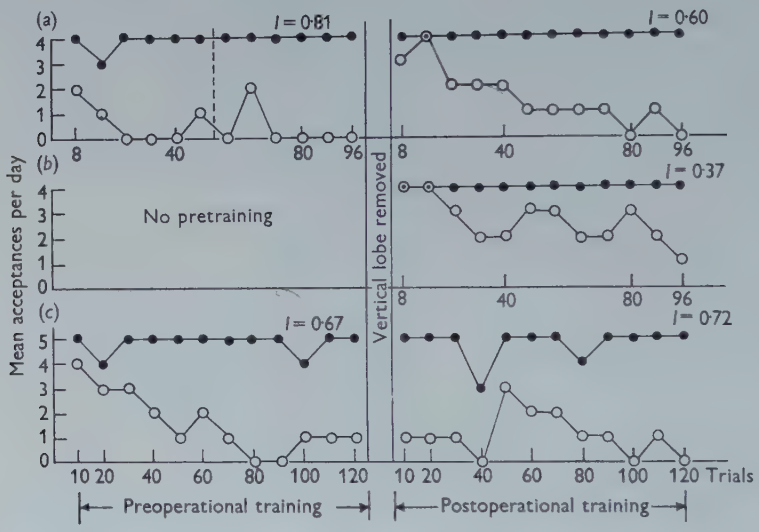

Fig. 1. Effects of a similar number of trials of preoperational training at 8 and 40 trials per day on the postoperational performance of animals following removal of their vertical lobes. (*a*) 3 NV animals; train–operate–train under the long-term conditions of 8 trials per day. (*b*) 6 NV animals; operate–train under the same conditions. (*c*) 3 NV animals; train-operate-train under the short-term conditions of 40 trials per day. ●, positive object; ○, negative object. Vertical dotted line indicates break in training of 36 hr. There was a similar break after vertical lobe removal.

These results show that some effects of both long- and short-term pretraining survive vertical lobe removal, and thus confirm the results of retention tests (Tables 3 and 4) showing postoperational survival of memories ensuring distinct responses to the test objects after long- or short-term pretraining.

Whatever changes to the structure or organization of their nervous systems have occurred as a result of training, these changes are not limited to the vertical lobe. If this is so, why are the responses of these animals less accurate after vertical lobe removal? We can show, by using animals operated without pretraining, that under suitable conditions those parts of the nervous system outside the vertical lobe are capable of forming and maintaining memories ensuring an accuracy of response equal to that of unoperated animals, at least in simple discriminations, although rather more trials are required to reach this state than when the vertical lobe is present (Wells & Wells, 1957*a*). When this is considered, together with the results

of the present series of experiments, which show a partial loss of learned responses after operation and subsequent recovery when training is continued, several possible ways in which the vertical lobe may be contributing to the learning process can be envisaged.

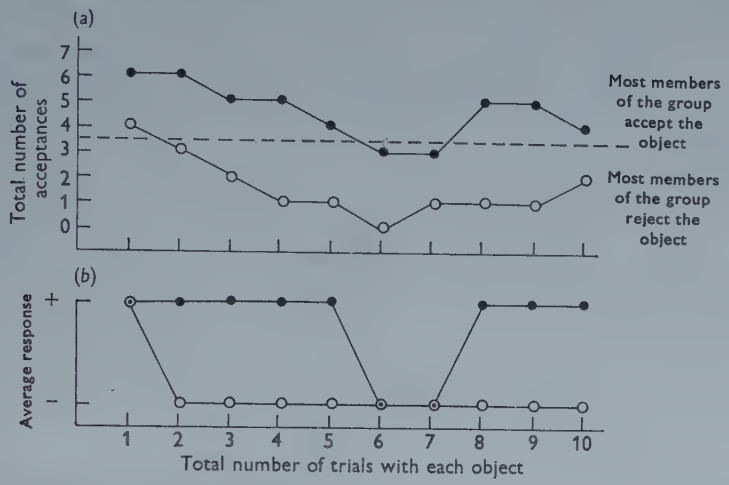

Fig. 2. Detail of the results of the retention tests with operated animals shown in Table 4. Each retention test consisted of 20 trials in the order + − + − + + − − + − + − − − + + − + − + −. ●, indicates positive; ○, the negative object. (*a*) gives the total number of times that the seven animals tested took each object at each trial. The ratio of positive to negative objects taken by the group remained about the same throughout the 20 trials of the retention test, although the total number of objects taken at each trial became less as the test progressed. (*b*) gives the same results considered in an all or nothing manner (i.e. whether the group, considered as a whole, reacted positively or negatively to the object presented at each trial). It can be seen that a distortion of the true result is produced, giving the impression that the animals failed to discriminate in some trials and succeeded in others, whereas (*a*) clearly shows that the animals discriminate between the objects to about the same extent throughout the experiment. Analogous distortions are inherent in discrimination experiments in which the degree to which animals can separate objects is concealed because they must react to them in an all or nothing manner (see text).

Previous workers have drawn attention to the fact that in many experiments vertical lobe removal is followed by an increase in the number of attacks made in situations that the animals had learned to avoid preoperationally. Sutherland (1957) has suggested that in visual discriminations the vertical lobe has a mainly inhibitory function, serving to keep a balance between positive and negative responses that is lost when this part is removed. Boycott & Young (1956, 1957) and Young (1956), again on a basis of the results of visual experiments, take the view that the vertical lobe is not so much concerned with an over-all inhibition of the 'positiveness' of response as with the formation of memories preventing attack. On the face of it, these views are supported by the tactile experiments described in this account, in all of which there is seen a tendency to err by reacting positively towards all the objects touched after vertical lobe removal. On the other hand, an exactly similar condition is found in *untrained* animals, which typically take all objects presented to them at the start of tactile training experiments (Wells & Wells, 1956, 1957*b*), and

in trained animals subjected to retention tests after breaks in training of from 5 to 10 days (Wells & Wells, 1958). Moreover, the ratio of errors made by accepting negative objects to errors by rejection of positives is found to be similar in controls and in animals lacking the vertical lobe, both of which err predominantly by acceptance of the negative objects in tactile training (Wells & Wells, 1957*a*). Taken together, these facts indicate that the tendency to positive responses following removal of the vertical lobes in tactile experiments may be attributable to a lack of effective memories of any sort rather than to a lack of memories specifically connected with negative responses.

The present authors do not believe that the facts so far available justify the assumption that the vertical lobe has a different function in the case of positive and negative memories, and suggest that its presence merely serves to increase the mass of nervous tissue available for memory retention without in any way altering the sort of memories that can be retained. If this is so, then its removal might be expected to obliterate 'positive' and 'negative' memories equally.

The nervous system of a single animal may be regarded as a population of neural units whose individual effects summate to produce the responses we observe. If the conditions of a discrimination experiment dictate that responses must be either 'positive' or 'negative', the results obtained can only record the condition of the animal's nervous system in a form such as that given in Fig. 2*b*, which is necessarily a distortion of the truth. When this is taken into account, apparently paradoxical results like those obtained by postoperational retraining and retention tests become comprehensible. In the experiments in Table 1, where long-term training was continued after operation, the animal at first took all the objects, which could be interpreted as showing that no memories permitting separation of the objects survive the operation. Yet when retention tests are carried out after operation instead of continued training, clear evidence that such memories do survive the operation is obtained. To explain this we need only suppose that the animal's positive responses to the negative objects are in some way less positive than its responses to the positive objects in both experiments. This difference is not at first revealed under the training conditions in which rewards and punishments are given, possibly because feeding the animals tends to raise their tendency to react positively as Young (unpublished) has shown in visual experiments. Under the retention test conditions, however, the animals' tendency to react positively to objects of both sorts is lowered as a result of repeating unrewarded actions until the weaker positive response to the negative object becomes ineffective.*

There is naturally no sharp transition between weak and strong memories, and in experiments in which attempt has been made to rate the degree of reaction as, for example, by timing the delay before attacking in a repeated and rewarded situation (Boycott & Young, 1950, 1956), it has been shown that the delay before attacking

* When a series of retention tests is made with an animal that at first reacts positively towards both of the objects, it is usual to find a progressive fading of positive responses, first to the negative and then to the positive object. In such series it is therefore usual to find a reduction in the proportion of errors made over the first few groups of trials (animal takes fewer negatives), followed by an increase as the animal begins to reject both objects (Wells & Wells, 1958).

decreases steadily as memories of specific situations become better and better established. The strength of a memory—as rated by the increase in probability of a particular type of reaction in a particular situation as a result of experience—will depend upon the cumulative effects of past experience and the time elapsed since the last occurrence of that situation. A memory becomes established throughout considerable masses of nervous tissue in octopuses as in vertebrates (Boycott & Young, 1950; Lashley, 1950) and it seems reasonable to suppose that the effectiveness or strength of a memory depends upon the total mass of cells or units available. Removal of a proportion of these would produce a proportional weakening of the memory concerned, making it statistically less likely to influence motor reactions to stimuli in any particular way. The authors regard removal of the vertical lobe as reducing the amount of memory-containing tissue in this way. Memories acquired as a result of preoperational training become weakened, and residual memories left elsewhere in the nervous system can only be revealed by altering the state of the animal as has been described above. The effect of further training summates with these residual (sometimes in themselves ineffective) memories, and in long-term discrimination experiments it is only possible to detect their postoperational survival by comparing the performance of pretrained and unpretrained animals. It should be noted, however, that because the capacity of the whole system has been permanently reduced by the operation, the standard of accuracy of responses may never reach that attained preoperationally. This is likely to be particularly evident in the case of difficult discriminations—those in which even the best established memories scarcely permit controls to separate situations to be discriminated with regularity—so that one would expect to find a relation between the difficulty of the most difficult discrimination that can be learned and the proportion of vertical lobe remaining under any given set of training conditions. Evidence from visual and tactile discrimination experiments indicates that this is so (Boycott & Young, 1957; Wells & Wells, 1957*a*). Since the strength of a memory depends among other things upon the length of time since it was last reinforced, (*a*) the performance of animals in any given discrimination and (*b*) the most difficult discrimination that an animal can be taught to make will also depend upon the interval between trials, so that it should prove possible to compensate for vertical lobe removal to some extent by placing trials closer together. This has also been demonstrated in discrimination experiments (Wells & Wells, 1957*a*).

SUMMARY

1. Blind octopuses were trained to discriminate between two objects by touch by taking one and rejecting the other. When they had learned to do this their vertical lobes were removed and postoperational survival of the effects of preoperational training was tested, either by continuation of training or by means of retention tests.

2. When training was continued after vertical lobe removal animals pretrained at a rate of 8 trials per day for 48 or 96 trials reverted to taking both of the objects

to be discriminated (as at the start of training), but subsequently relearned to discriminate between them with an accuracy approaching that of controls. They took fewer trials to learn after operation than animals that had not been pretrained.

3. Animals pretrained at a rate of 40 trials per day for 120 trials showed little or no disturbance of learned responses as a result of the same operation.

4. In retention tests carried out immediately after operation, animals pretrained for a similar number of trials at rates of 8 and 40 trials per day made more errors than controls, but showed, nevertheless, that the effects of pretraining by either method were not entirely lost as a result of the operation.

5. These results are discussed in relation to the general problem of the interpretation of discrimination experiments which force animals to react 'positively' or 'negatively' in an all-or-nothing manner and thereby conceal differences in the degree to which memories are established.

6. It is concluded that the effect of vertical lobe removal can be attributed to a reduction in the amount of tissue available for memory retention.

Most of the experiments reported in this paper were made in 1954–56, while M.J.W. was holding an Eli Lilley Fellowship as a member of the staff of the Stazione Zoologica di Napoli, the rest during a visit to the Stazione in the summer of 1957 while holding a Fellowship at Trinity College, Cambridge. The authors would like to thank Prof. J. Z. Young, F.R.S., and Mr B. B. Boycott, who have kindly read and criticized this work in manuscript, and the Director and Staff of the Stazione Zoologica for their hospitality to an ex-colleague during 1957.

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THE EFFECT OF VERTICAL LOBE REMOVAL ON THE PERFORMANCE OF OCTOPUSES IN RETENTION TESTS

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INTRODUCTION

Removal of the vertical lobe from the brain of blind octopuses causes defects in the performance of these animals under training conditions. When such animals were trained postoperationally to make tactile discriminations known to be made readily by blind but otherwise unoperated animals they made many more errors than controls. The effect of vertical lobe removal upon the number of trials required to train octopuses to a given criterion of response was more marked when trials were at intervals of 1 hr. than when trials were at intervals of 5 min., the performance of animals under the latter conditions approaching that of controls (Wells & Wells, 1957*a*). It was concluded that the vertical lobe contributes to the persistence of memories between trials; a similar interpretation of the results of visual discrimination experiments was made by Boycott & Young (1955, 1957). Further experiments, in which the behaviour of animals in the *first* trial of training experiments was particularly considered, showed that removal of the vertical lobe has effects upon the performance of animals that cannot be attributed to failure to retain memories between trials (Wells & Wells, 1957*b*). On a basis of the results of the latter experiments it was suggested that the vertical lobe serves to increase the effect of sensory experience in the establishment of memories during the period in which the sensory input is actually occurring rather than to reduce the rate of decay of memories once set up.

If this were so one would expect to find that memories once established would fade at about the same rate in controls and in animals lacking their vertical lobes. The present account is of retention tests made with controls and 'no vertical' animals that have previously been trained to the same standard of accuracy of response.

MATERIAL AND METHOD

Octopuses of from 250–750 g. from the Bay of Naples were obtained and treated as described by Boycott (1954). Before use in training experiments all the animals were blinded by section of their optic nerves and some of them were subjected to a further operation in which parts of the brain were cut out. Details of operational methods are given in Wells & Wells (1956, 1957*a*). After a postoperational period of several days, during which the octopuses were fed upon pieces of fish but given

no experience of the test objects, training experiments were carried out as described in Wells & Wells (1957*a*).

In these experiments animals were required to learn to distinguish by touch between the members of a pair of Perspex cylinders, one of which was smooth (P4), the other (P1 or P8*) roughened by deep grooves cut into it. Training consisted of a number of trials, at each of which one or other of the objects was presented, the animals being rewarded with a piece of fish for passing the 'positive' object to the mouth, and punished with a small (6 V. a.c.) electric shock if they did the same with the 'negative'. There were 40 such trials per day, in two groups of 20 (10+, 10−) each systematized thus:

+ − + − + + − − + − + − − + + − + − + −.

Individual trials in a group were at intervals of 5 min. and the start of the second group of trials on any day was not less than 6 hr. after the beginning of the first. Under these conditions octopuses speedily learn to discriminate between the test objects, passing the positive object to the mouth and rejecting the negative by thrusting it away to arm's length whenever presented.

For the present series of experiments animals were trained until they attained a standard of either 75% or 85% correct responses (five and three errors or less respectively) in a group of 20 trials and were then overtrained for a similar number of trials. During the period between the cessation of training and the start of retention tests the animals were fed regularly but given no contact with the test objects. No other experiments were made during these periods, so that the animals had no opportunity of tactile experience other than that afforded by their food or contact with the surfaces of their aquaria. Retention test trials were carried out in groups of twenty exactly as in training except that no rewards or punishments were given.

Throughout this account individual animals are referred to by the number with which they were identified in our original protocols. This number is made up of a prefix (B, C or D being 1955, 1956 and 1957 respectively), an individual reference number and a suffix indicating the lesion made. The suffixes appearing here are B = blind (optic nerves cut), NOL = no optic lobes, NV = no vertical lobe, and NSF = no superior frontal lobe. Thus C 10 NVNSFB indicates a blind animal with a lesion to the vertical and superior frontal lobes, experiment made in 1956. The extent of lesions made was measured from serial sections as described in Wells & Wells (1957*a*).

The vertical lobe lies immediately behind and in some places beneath the superior frontal lobe (Young, 1951) so that it is difficult to ensure complete removal of the former without some damage to the latter. In visual experiments damage to the lateral parts of the superior frontal lobes appears to prevent octopuses from attacking objects seen at a distance, making it difficult or impossible to train them (Boycott & Young, 1955). No such undesirable effects are produced by comparable operations in tactile experiments where the performance of animals with both the

* P8 had more grooves and was therefore rougher than P1. Octopuses find the P1/P4 discrimination about twice as difficult as the P8/P4 (Wells & Wells, 1957*c*). In the present series of experiments all the animals save those listed in Table 4 were trained to discriminate between P1 and P4.

superior frontal and the vertical lobes removed appears to be indistinguishable from that of animals lacking the vertical lobe only (Wells & Wells, 1957*a*, 1958). In most of the operations made for the present series of experiments part of the superior frontal lobe was cut away to ensure complete removal of the vertical lobes.

RESULTS

Because of the considerable number of animals involved, it is not practicable to publish details of the training of all the octopuses used in the retention tests reported in this account. The performance of some typical individuals has, however, been recorded in full elsewhere, and references are included in the tables where records of such animals appear.

(1) *Experiments with control animals*

Among the blind controls are included two animals with the optic lobes removed by section of the optic tracts peripheral to the optic tract ganglia; this operation has been shown elsewhere to be without effect on tactile learning (Wells & Wells, 1957*a*). The results of retention tests with these controls are given in Table 1. This table shows that six animals (group A) first tested 5 days after the cessation of training averaged 3·2 errors in a group of 20 trials compared with less than one error in their last 20 training trials. Five of these animals tested again 10 days later averaged 5·2 errors. A second group of four animals (group B), trained to a criterion of 75% correct responses instead of the usual 85%, averaged 6·8 errors when first tested for retention at 10 days. In both groups the range of variation (of from 1 to 10 errors) was very great. It should be noted that with one exception (D 11 B, 10-day test) animals erred in retention tests predominantly by taking the objects that they had previously been trained to reject. Errors due to failure to accept 'positive' objects were comparatively rare. It has already been pointed out elsewhere (Wells & Wells, 1956, 1957*c*) that in the absence of punishment octopuses tend to revert to taking all small objects that they touch, including those that they have previously been taught to reject.

It is generally possible to demonstrate that such animals still retain memories enabling them to discriminate between objects that they have been taught to distinguish. This is most readily achieved by repetition of retention tests at frequent intervals until the animal ceases to repeat the unrewarded action of taking the objects. It is found in such cases that the positive response to the 'negative' object disappears before the positive response to the 'positive' object, so that there is a transitory improvement in performance during which the animal may make no errors at all.

The process of revealing latent discriminatory memories in retention tests can be accelerated by giving small electric shocks, as used in training, either at each trial (regardless of whether the object presented has been 'positive' or 'negative' in pretraining), or in a group shortly before the beginning of the retention test. An example is given in Fig. 1. This animal (D 3 3 B) made only 1 error in a retention test 5 days after the end of its training, but failed to discriminate at all in a similar

test 10 days later, taking all the objects that were presented. Six hours later the animal was retested, but this time a small (6 V. a.c.) electric shock was given immediately after each of the first 8 trials (4 with the positive object and 4 with the negative). As a result of this treatment only the first two negative objects were taken. After eight shocks the animal began to reject also the positive objects and no further shocks were given, with the result that positive responses to the positive objects but not to the negative objects were resumed.

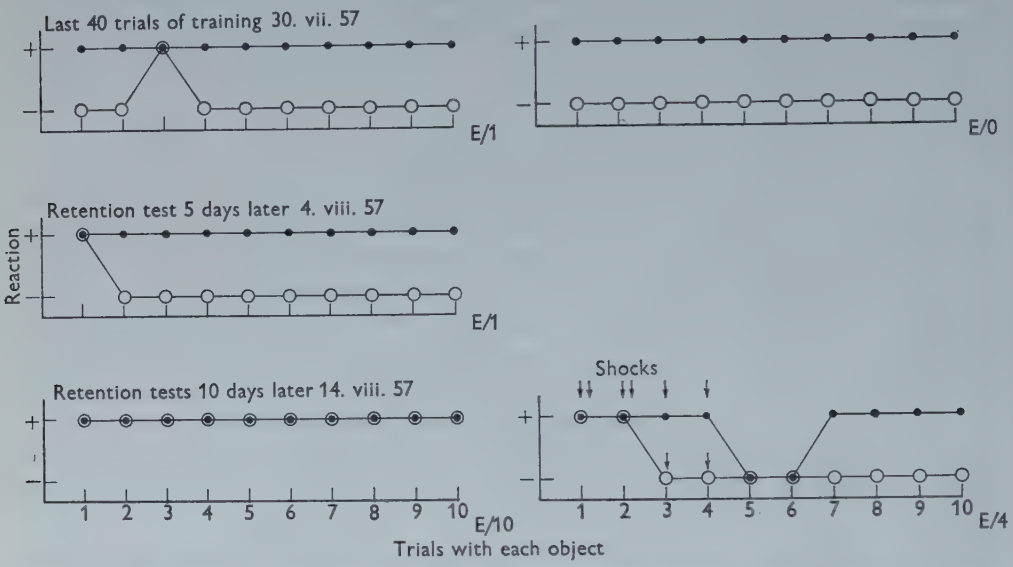

Fig. 1. Detail of the results of a typical series of retention tests; octopus D 33 B. The performance of the animal in the last 40 of 120 training trials is given in full; reactions are recorded as + when the animal accepted the object and as − when it rejected it. Training was followed by an unrewarded retention test 5 days after the end of training, and again 10 days later. In the first set of trials at the end of the second period, the animal took all the objects presented. In a second series 6 hr. later small electric shocks, indicated ↓, were given at the first 8 trials (regardless of the object presented and the animal's reaction to it); this lowered the animal's tendency to respond positively and revealed persistence of the ability to discriminate between the objects.

Other instances where a series of retention tests was made in quick succession, with or without electric shocks, are recorded in Tables 3 and 4. There is a considerable individual variation in the number of trials required to eliminate responses to the test objects but all such series show the same cycle of events (positive response to the negative object fades before the positive response to the positive) as D 33 B.

Results of this sort are perhaps best interpreted as revealing memories of the negative object that are too 'weak' to influence the type of reaction made when the animal is otherwise likely to respond in a strongly positive manner towards objects that it touches. When this tendency is reduced by frequent repetition of unrewarded actions, or by electric shocks, memories at first masked are revealed (Wells & Wells, 1958). Feeding, on the other hand, increases the probability of positive

reaction. In Tables 3 and 4 there are five instances in which animals were fed shortly before retention tests. In four out of these five cases the proportion of objects taken was greater than in the last tests before feeding (normally animals were fed immediately after the completion of each section of tests, so that they were never tested less than four hours after last being fed). In the remaining instances the proportion of objects taken remained the same. Similar effects have been shown in visual experiments where feeding shortly before tests enhances the tendency to react positively and may conceal memories that would certainly lead to discrimination if the animals remained unfed (Young, 1957).

Table 1. *Retention tests with control animals trained to discriminate between P1 and P4*

(Each test consisted of 20 trials, 10+, 10−. Columns show the number of times that each object was taken and the total errors (column E).)

	In the final 20 trials of training			In retention tests						
				Time after end of training or last test						
				5 days			10 days			
	+	−	E	+	−	E	+	−	E	
Group A. Animals trained to a criterion of 85 % correct responses before overtraining										
C158B	10	0	0	10	4	4	.	.	.	.
D11B	10	0	0	8	2	4	1	0	9	.
D16B	10	1	1	10	3	3	10	2	2	For subsequent tests see Table 3
D19B	10	0	0	10	2	2	10	1	1	.
D26B	10	1	1	10	5	5	9	3	4	.
D33B	10	0	0	10	1	1	10	10	10	For details see Fig. 1
	.	.	.	.	.	.	8	2	4*	.
Mean errors		1·4			3·2			5·4 (4·0)*		.
Group B. Animals trained to a criterion of 75 % correct responses before overtraining										
C2B	10	0	0	.	.	.	8	3	5	.
C4B	6	0	4	.	.	.	10	10	10	For details of training see Wells & Wells (1957*a*)
	.	.	.	.	.	.	9	5	6*	
C6NOL	9	2	3	.	.	.	10	5	5	.
C19NOL	9	1	2	.	.	.	10	7	7	.
	.	.	.	.	.	.	3	0	7*	.
Mean errors		2·3		.	.	.		6·8 (5·8)*		.
	.	.	.	.	.	.				.

* Tests 6 hr. later in which 6 V. a.c. electric shocks were given after each of the first eight (D33B) or ten (C4B, C19NOL) objects presented regardless of reaction or whether the objects were + or −. Averages in brackets are calculated using scores from these retests. Where both 5- and 10-day retention tests were made, the 5-day always preceded the 10-day.

These possible masking effects must be taken into account if meaningful comparisons are to be made between the performances of individual octopuses in retention tests. In particular, it must be borne in mind that an animal taking all of the positive objects and a high proportion of the negatives is unlikely to be separating the objects with the maximum accuracy of which it is potentially capable. In

Table 1, for example, the score of D 26B in its 10-day retention test (took 9 positives and 3 negatives, errors 4) is probably a reliable index of this animal's ability to separate the test objects at that time, whereas the score of D 33B at 10 days (took everything) is manifestly not (see Fig. 1). When the results given in Table 1 are considered in this way it is evident that the retention test results obtained with C 4B and D 33B after shocks are fairer assessments of the persistence of discriminative memories than the figures obtained in the immediately preceding straightforward retention tests.

Table 1 therefore shows that although fading of discriminative memories has undoubtedly occurred during the 10 (group B) or 15 (group A) days since the end of training, in no case have the memories altogether disappeared, and that most individuals remain capable of discriminating between the objects with an accuracy of better than 75 % (15 correct out of every 20 responses) for at least this long.

(2) *Experiments made with animals after vertical lobe removal*

Table 2 shows the results of exactly similar retention tests made with 18 animals having brain lesions that in most cases included total removal of the vertical lobe. Eleven of these animals averaged 4·7 errors (4·1 when a retest with C 144 NVB is taken into account) in retention tests carried out 5 days after the end of their training, and six of these averaged 5·4 errors after a further 10 days. In the last 20 trials of their reward and punishment training the same eleven animals averaged 2·8 errors. A second group of seven animals pretrained to a criterion of only 75 % correct responses averaged 7·4 errors in tests 10 days after the end of their training (or 6·9 errors using retests), compared with 2·9 errors in the last 20 training trials.

These results are clearly similar to those reported in Table 1, considerable traces of discriminatory memories remaining apparent 10 days after the end of training. As with controls discriminatory memories can generally be revealed in animals initially taking a high proportion of both objects by subjecting the animals to repeated retention tests or to small electric shocks which reduce the tendency to react positively (Tables 2 and 3). In both cases positive responses to the negative object are eliminated before positive responses to the positive so that there is typically a transient improvement in performance as tests are continued (see also Table 4).

(3) *Length of memory retention in* Octopus

In the present investigation no systematic attempt has been made to find out how long memories of learned discriminations persist in the nervous system of *Octopus*, but in several instances retention test series were continued intermittently for a considerable time after cessation of training without entirely eliminating the tendency to react differentially towards the two test objects. Thus D 11B made 8 errors in a group of 20 trials 20 days after the last test shown in Table 1—a total of 35 days from the end of training; B 164 NVB made only 5 errors in a retention test after the same total length of retention time (see Table 3). B 161 NVNSFB and C 41 NVB made only 4 and 6 errors respectively at 27 days, while B 16B and C 45 NVB made 6 and 8 errors 24 and 26 days after the end of training. It should be

Table 2. *Retention tests with animals having brain lesions trained to discriminate between P1 and P4*

(Each test consisted of 20 trials, 10+, 10−. Columns show the number of times that each object was taken and total errors (column E).)

Animal and proportion of the vertical lobe removed	In the final 20 trials of training			In retention tests: Time after end of training or last test: 5 days			10 days			
	+	−	E	+	−	E	+	−	E	
Group A. Animals trained to a criterion of 85 % correct responses before overtraining										
C36NVNSFB (100 %)	10	2	2	9	1	2	4	2	8	.
C37NVNSFB (100 %)	2	0	8	10	3	3	9	1	2	10 days later 0, 0, 10
C41NVNSFB (100 %)	9	0	1	10	2	2	10	1	1	For later tests see Table 3
C45NVNSFB (100 %)	7	0	3	9	3	4	.	.	.	20 days later 7, 5, 8
C49NVNSFB (100 %)	5	0	5	2	0	8	3	0	7	.
C51NVB (100 %)	10	2	2	8	1	3	4	0	6	.
C144NVB (85 %)	10	1	1	10	8	8	2	0	8	.
	.	.	.	10	1	1*	.	.	.	
C173NVNSFB (100 %)†	10	1	1	10	4	4	.	.	.	For later tests see Table 3
C188NVNSFB (100 %)†	10	2	2	10	4	4	.	.	.	
C189NVB (70 %)†	7	2	5	10	10	10	.	.	.	.
C196NVNSFB (100 %)	10	1	1	8	2	4	.	.	.	.
Mean errors		2·8			4·7 (4·1)			5·4		.
Group B. Animals trained to a criterion of 75 % correct responses before overtraining										
B158NVNSFNOL (100 %)‡	10	2	2	.	.	.	1	1	10	Extract from a series of tests given in full in Table 3
B161NVNSFB (100 %)‡	9	1	2	9	4	5	10	7	7	
B164NVB (90 %)‡	10	5	5	10	1	1	10	1	1	
C9NVNSFB (100 %)‡	10	3	3	.	.	.	6	2	6	.
C10NVNSFB (100 %)‡	10	2	2	.	.	.	10	9	9	.
	.	.	.	.	.	.	8	3	2§	.
C11NVB (100 %)‡	10	3	3	.	.	.	10	9	9	.
C16NVNSFB (100 %)‡	8	1	3	.	.	.	8	6	8§	.
Mean errors		2·9			3·0			7·4 (6·9)		.
	.	.	.	.	.	.				.

* Second set of tests on the same day, no shocks.

† Details of pretraining given in Wells & Wells (1958). Where both 5- and 10-day retention tests were made the 5-day always preceded the 10-day test.

‡ Details of pretraining and of lesions given in Wells & Wells (1957*a*).

§ Tests 6 hr. later in which 6 V. a.c. electric shocks were given after each of the first 10 objects to be presented, whether + or −. Averages in brackets are calculated using scores from these retests.

remembered that all these animals had been subjected to retention tests since the end of training and that the effect of these tests, involving frequent repetition of unrewarded actions, is to eliminate responses to the test objects (Wells & Wells, 1957*b*). The survival of distinct responses to the test objects, despite this treatment, surely indicates that memories normally persist for considerably longer, perhaps for a period of months. Sutherland (1957) reports a retention experiment in which six animals trained to distinguish between vertical and horizontal rectangles by sight showed no decline in performance when tested 27 days after the end of

Table 3. *The proportion of acceptances, first of negative and then of positive objects, is successively reduced by repeated retention tests*

(The sequence of tests reads down the columns, which show the number of times that each object was taken and the total errors made (column E) in each group of 20 trials. Tests on the same day (bracketed together) were 6 hr. apart, otherwise overnight intervals of approximately 18 hr. except where specified. The initial interval in each case is the time since the end of training. Figures in parentheses give the proportion of vertical lobe removed. Controls and animals in column A were trained to a criterion of 85 % and those in column B to 75 % correct responses before overtraining.)

Controls				Animals with brain lesions							
				A				B			
	+	−	E		+	−	E		+	−	E
D16B	5 days			C41NVNSFB (100 %)	5 days			B158NVNSFNOL (100 %)	5 days		
	10	3	3		10	2	2		4	0	6
	10 days				10 days				4	1	7
	10	2	2		10	1	1		3	0	7
	10	2	2						10 days		
	10	0	0		10 days				1	1	10
	10	1	1								
10 shocks 20 min.	10	0	0		10	10	10				
before test	10	0	0	Shocks after first 10 presentations	10	8	8	B161NVNSFB (100 %)	5 days		
10 shocks 10 min.	8	0	2		10	2	2		9	4	5
before test	10	0	0	With all 20	10	6	6		10	5	5
	10	0	0						10	4	4
	9	0	1								
	3	0	7	C173NVNSFB (100 %)	5 days				11 days		
	4	0	6		10	4	4		10	7	7
	5	0	5		8	2	4				
	5	0	5		3	0	7		11 days		
	2	1	9						8	2	4
	5	0	5								
	2	0	8								
Fed 30 min.	3	0	7	C188NVNSFB (100 %)	5 days			B164NVB (90 %)	2 days		
before test	4	0	6		10	4	4		10	1	1
					10	3	3				
D19B	5 days				10	0	0		5 days		
	10	2	2		9	1	2		10	1	1
					1	0	9				
	10 days								6 days		
	10	1	1	C189NBV (70 %)	5 days				10	0	0
	2	0	8		10	10	10		10	5	5
Fed 45 min.	2	0	8		10	10	10		10	0	0
before test					10	10	10				
					10	10	10		11 days		
					2	0	8		10	1	1
D33B	See Fig. 1										
									11 days		
				C196NVNSFB (100 %)	5 days				10	5	5
					8	2	4				
					9	4	5				
					3	0	7				
					5	1	6				
					0	0	10				

training; these animals had, however, been trained in the meantime to distinguish between the same two rectangles in oblique positions. There are no other records of experiments made to test visual retention by *Octopus* over comparable periods.

Table 4. *Animals subjected to retention tests after operations following training to discriminate between P8 and P4*

(Training was at a rate of 8 trials per day (4+, 4−) for 6 days. Retention tests were made at the normal rate of 40 trials per day, in groups of 20. Conventions as Table 3.)

Control (dummy operation only)				Animals with brain lesions							
	+	−	E		+	−	E		+	−	E
D9B	36 hr.			D1NVB (80%)	36 hr.			D2NVNSFB (100%)*	36 hr.		
	10	0	0		10	6	6		10	9	9
	10	0	0		9	1	2		5	4	9
	9	0	1		1	0	9		4	2	8
	6	0	4		5	0	5		2	1	9
	5 days				3	3	10		1	0	9
	7	0	3		5 days						
	4	0	6		5	10	15	D8NVNSFB (100%)	36 hr.		
	0	0	10		1	7	16		8	2	4
Fed 30 min. before test	1	0	9						7	1	4
				D4NVB (90%)	36 hr.				10	1	1
					10	3	3		10	0	0
					9	1	2		8	0	2
					5	0	5		5 days		
					2	0	8		10	1	1
					1	0	9		10	0	0
					5 days				7	1	4
					2	1	9		2	0	8
					0	1	11	Fed 30 min. before test	9	0	1
									6	0	4
								Fed 60 min. before test	10	0	0

* In this animal the superior frontal lobe was entirely removed. This disconnects the vertical lobe from its tactile input (Boycott & Young, 1955) so that for purposes of comparison this animal may be regarded as having the vertical lobe entirely removed, although a part of the extreme right hand gyrus of this lobe (about 15% of the total volume of the lobe) remained intact after the operation.

DISCUSSION

The object of the present series of experiments is to establish whether or not memories of things touched fade more rapidly in animals from which the vertical lobe of the brain has been removed. The records of retention tests with 'no vertical' and control animals clearly show that there are no gross differences in the performances of the two groups of animals. Both are subject to the same tendency to revert to positive responses in the absence of tests, and both can be shown to retain effective discriminatory memories for at least 10 days after cessation of training.

The performance of control and 'no vertical' animals is compared in greater detail in Table 5. It is apparent from this that the system of training animals to

Table 5. *Summary of results of retention tests*

	In final 20 trials of training	In retention tests	
		Time after end of training or last test	
		5 days	10 days
(a) Mean errors per 20 trials			
Groups A. Animals trained to a criterion of 85 % correct responses before overtraining			
Controls	1·4	3·2	5·2 (4·0)
Animals with vertical lobe lesions	2·8	4·7 (4·1)	5·4
Groups B. Animals trained to a criterion of 75 % correct responses before overtraining			
Controls	2·3	.	6·8 (5·8)
Animals with vertical lobe lesions	2·9	.	7·4 (6·6)
(b) Increase in errors per 20 trials			
Groups A. Animals trained to a criterion of 85 % correct responses before overtraining			
Controls	.	1·8	3·8 (2·6)
Animals with Vertical lobe lesions	.	1·9 (1·3)	2·6
Groups B. Animals trained to a criterion of 75 % correct responses before overtraining			
Controls	.	.	4·5 (3·5)
Animals with vertical lobe lesions	.	.	4·5 (3·7)

(Figures in parentheses are calculated using scores made in retests (see p. 339).)

a prescribed criterion of accuracy regardless of the length of training required has failed to achieve its object of ensuring the same standard of accuracy of response in control and 'no vertical' animals before retention tests. All of the animals attained the prescribed criterion (85 or 75% correct responses in a group of 20 trials), although those lacking the vertical lobe were slower to do this (Wells & Wells, 1957*a*), but improvement thereafter during overtraining was greater in controls than in the octopuses lacking vertical lobes. It had been hoped to avoid this by overtraining each individual for a number of trials equal to that needed to reach the prescribed criterion, but the attempt was unsuccessful, and one must conclude that the presence of the vertical lobe becomes relatively more important in the later phases of learning when the accuracy of a response is in the process of refinement towards the optimum of which the animal is capable. This finding is consistent with what we know about the relatively greater effect of vertical lobe removal upon the performance of octopuses in difficult discriminations (Wells & Wells, 1957*a*; Boycott & Young, 1957).

So far as the present series of tests is concerned this finding means that animals lacking the vertical lobe begin their retention tests with memories rather less well established than their controls. The process of forgetting in the two preparations must be studied relative to their respective starting-points. In the case of group A controls, for example, the 10-day retention test figure of 5·2 errors (4·0 when retests are considered) must be compared with a starting accuracy of 1·4 errors per 20 trials, and the 5·4 of the 'no verticals' with 2·8. This has been done in Table 5*b*, which shows the increase in number of errors made per 20 trials in successive retention tests; almost exactly similar figures are obtained from experiments with controls and 'no vertical' animals. In view of the wide range of individual scores made in these tests and the rather small number of animals involved (28 in all) it would be unwise to pretend that the experiments summarized in Table 5 show conclusively that memories fade at the same rate in animals with and without vertical lobes. The results do, however, suggest very strongly that this may be the case, and in any event give no grounds for supposing that any considerable difference in the rate of memory fading is produced by vertical lobe removal.

These results are consistent with the hypothesis that at least in so far as touch is concerned the vertical lobe plays a part in the establishment of memories during the time that the sensory stimuli concerned are being received but not in the maintenance of these memories between trials. Its removal renders each sensory experience less effective in the establishment of memories, but does not affect the rate at which whatever changes have occurred in the nervous system fade away between such events (Wells & Wells, 1957*b*).

SUMMARY

1. Octopuses were trained to make a tactile discrimination until they attained a criterion of either 75% or 85% correct responses in twenty successive trials. They were then overtrained for an equal number of trials. Retention of training was tested in twenty unrewarded trials with the same objects 5 or 10 days later.

2. Exactly similar experiments were made with animals trained after removal of the vertical lobes of their brains; these animals were slower to learn, but once trained to a similar standard of accuracy of response they forgot their training at the same rate as controls.

3. This shows that while the presence of the vertical lobe enhances the effect of experience on the establishment of memories causing discrimination of things touched, it does not affect the maintenance of these memories once established.

The experiments listed in this account were made during the period 1955–6, while M.J.W. was holding an Eli Lilly Fellowship as a member of the staff of the Stazione Zoologica in Naples, and during a visit to the Stazione in the summer of 1957, while holding a Fellowship at Trinity College, Cambridge. The authors would like to thank the Director and Staff of the Stazione for their hospitality to an ex-colleague and to thank Prof. J. Z. Young F.R.S., and Mr B. B. Boycott for reading and criticizing this work in manuscript.

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TEMPERATURE ADAPTIVE BEHAVIOUR IN THE SCORPION, *OPISTHOPHTHALMUS LATIMANUS* KOCH

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In discussing the possible functions of the pectines of the scorpion, von Ubisch (1922) suggests that they serve 'um den Stigmen frische Luft zuzufuhren' by their fanning movements or, alternatively, they prop up the mesosoma so that fresh air can reach the stigmata. Both of these suggestions imply that under conditions of respiratory stress a scorpion will lift its mesosoma clear of the ground so that the book-lungs have free access to air. Although von Ubisch did not apparently observe such behaviour, the recent report of 'abdominal elevation' in the Australian scorpion, *Urodacus abruptus* Poc. (Southcott, 1955) lends colour to such an hypothesis, although work on the functions of the pectines makes it seem very improbable that they actually serve as 'respiratory fans'. Southcott gives an illustration of a female of *U. abruptus* in the attitude typical of 'abdominal elevating behaviour', and from a comparison of this with some of the stances observed in various South African scorpions it becomes clear that such behaviour occurs also in the latter. *Opisthophthalmus latimanus* Koch, *O. nitidiceps* Poc., *O. austerus* Karsch., *Parabuthus planicauda* Poc. and *Uroplectes triangulifer* Thor. all show the pattern to varying extents, and it was felt that with so many species available for study, it would be profitable to follow up Southcott's observations. Preliminary tests indicated that the biological significance of this phenomenon appeared to be the same in all these species, and the results described below refer particularly to *Opisthophthalmus latimanus* which has been most intensively studied.

In this animal the pattern generally does not consist of a simple elevation of the abdomen, but more usually of a raising up of the entire body of the scorpion by a straightening of all the legs. Fig. 1 illustrates semidiagramatically the differences between (*a*) the normal resting stance of *O. latimanus*, (*b*) the stance in which the whole body is lifted clear of the ground, and (*c*) the more extreme 'abdominal elevation'. There is indeed no sharp distinction between these last two, and other rather rarer variations occur, e.g. that in which the abdomen, lifted clear of the ground, is supported by the tail (Fig. 1*d*). All of these different stances have in common a straightening of the legs; they will collectively be referred to here as 'stilted poses' and the behaviour itself as 'stilting'. In none of the species studied here, nor in *Androctonus australis* (L.) (Cloudsley-Thompson, 1955), is there any indication that the pectines might be used to prop up the abdomen, as von Ubisch

has suggested. As in *Urodacus abruptus*, stilting occurs in both sexes of *Opisthophthalmus latimanus*, and it has been observed in all instars except the first where it might well not be recognizable.

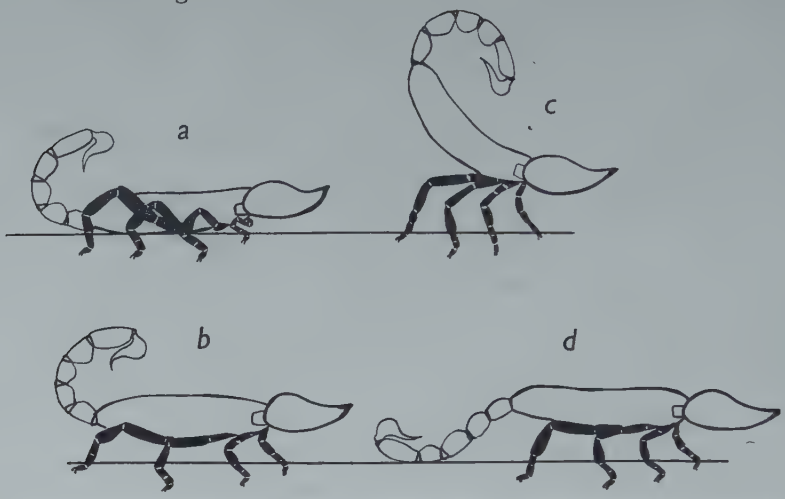

Fig. 1. *Opisthophthalmus latimanus*. Semi-diagrammatic representations of postures adopted. *a*, resting stance, venter to the substratum and legs folded; *b*, stilted pose in which the whole body is lifted away from the substratum by straightening the legs; *c*, stilted pose in which the mesosoma is sharply elevated; *d*, rather rare stilted pose in which the tail appears to help in propping the body away from the substratum.

FUNCTION OF THE STILTING

Working along the same lines as von Ubisch, Southcott (1955) states that stilting occurs in hot and humid conditions, and concludes that 'it would appear most likely that the stance...is an effort to lift the stigmata free from the humid layer of the air and soil, when the scorpion's metabolism is increased by a hot environment'. Cloudsley-Thompson (1955) has pointed out, with reference to the postulate of von Ubisch, that a scorpion has normally a very considerable respiratory margin of safety. For this reason it seems improbable that respiratory adaptive behaviour as such would be of importance in the life of a scorpion.

However, the effect of respiratory stress appeared to be worthy of further experimental investigation. The problem had already been studied in *Androctonus australis* by Cloudsley-Thompson (1955), using streams of gas composed of nitrogen and carbon dioxide. Observation of *Opisthophthalmus latimanus* has, however, shown that if a stilting animal is disturbed by a sudden draught of air, it drops its stilted pose and will often not resume it for some time. It was therefore considered desirable for observations to be made in still air. Furthermore, since extreme respiratory stress, such as that used by Cloudsley-Thompson, might itself inhibit an adaptive response, a series of gas mixtures was used: namely mixtures of air with approximately 20 or 75% carbon dioxide, and air with 60 or 90% nitrogen. In none of these mixtures were there the slightest signs of stilting. This was not due to the inability of the animals to stilt, for, if other suitable stimuli were applied, the

scorpions would stilt in these gas mixtures. Moreover, stilting was more easily elicited by these means in those gas mixtures containing the lower concentrations of carbon dioxide or nitrogen, a fact speaking against the importance of stilting in respiratory stress.

As a check, observations were made on scorpions in which the openings of the book-lungs had been blocked. This was done in one of two ways: either the mesosternites were smeared with vaseline, or the openings of the lungs were painted over with Gestetner Correcting Fluid or Samsonite. Observations lasting over 12 hr. showed no stilting by any of the animals, though the fact that two of the animals died shortly after indicates that their respiratory systems had probably been affected by the treatment. Controls with their backs painted all survived.

These experiments, together with the observations of Cloudsley-Thompson (1955) on *Androctonus australis*, make it unlikely that stilting has an adaptive significance in respiratory stress. To comprehend the phenomenon, it is therefore necessary to turn once more to observations of the conditions in which this pattern is shown.

Southcott (personal communication) states that in summer he need only pour a little water into his scorpion culture dishes to elicit stilting behaviour. Unlike *Urodacus abruptus*, *Opisthophthalmus latimanus* shows no such direct relationship between the humidity and stilting; stilting may occur when the scorpion is standing in a pool of water or, conversely, in a desiccator. The character of the substratum, whether it be smooth or rough, whether or not it be covered with chemicals distasteful to the scorpions, does not determine whether the animals will stilt.

However, although the pattern may occur at any time during the day or night, it is most frequently seen during the afternoon. This suggested that temperature might be a factor of importance in determining the onset of stilting. Confirmation of this can be obtained by observing the behaviour of animals in a dish of soil, the dish being warmed or cooled as desired. Below 18° C. stilting is not normally observed and if the soil on which a stilting scorpion is standing be cooled below this temperature, the animal's pose gradually reverts to the normal resting stance. Between 18° and 28° C. animals may or may not be found to be stilting, but at higher temperatures any animal that is standing still will usually be stilting to some degree, or may be reared up against the side of the dish, a condition amounting in effect to a stilted stance.

As mentioned earlier, *O. latimanus* does not stilt in direct response to the presence of water. Unlike the condition in *Urodacus abruptus*, there is no indication here that, with a higher humidity, the stilting behaviour is elicited at a lower temperature. This point was studied by trials on pairs of animals, one animal being kept in a dish at 40–60 % R.H.; the other at 85–90 % R.H. If anything, there was a slight indication that the animals reacted a little more quickly and at a slightly lower temperature when at the lower humidity.

Once it is clear that stilting is not concerned with respiratory adaptation, but is elicited merely by a high temperature, it is reasonable to ask whether the stilted pose may not have some thermoregulatory role. Drawing on the techniques used by

Colbert, Cowles & Bogert (1946) in their experiments on temperature adaptations in alligators, the following arrangement was used. Two live animals were fixed so that one, the experimental animal, could be held in a stilted pose while the other, the control, could be restrained in the normal resting position. In the absence of more complicated apparatus, abdominal temperatures were determined by clinical thermometers whose bulbs were inserted through a small lateral incision. In these experiments the scorpions were placed upon a copper plate lying on the ground. The plate was initially left in the sun to attain a uniform steady temperature. A small shade was then placed so as to screen the area of plate upon which the two animals would be placed, and the scorpions were then quickly put in position and their abdominal temperatures recorded. After 10 min. their temperatures had not changed; the shade was then removed so that the sunlight fell on the animals and the observations on their temperatures were continued. Preliminary experiments showed that the orientation of the animals towards the sun was not critical, and in all those reported below the scorpions were oriented with their longitudinal axes at right angles to the sun's rays.*

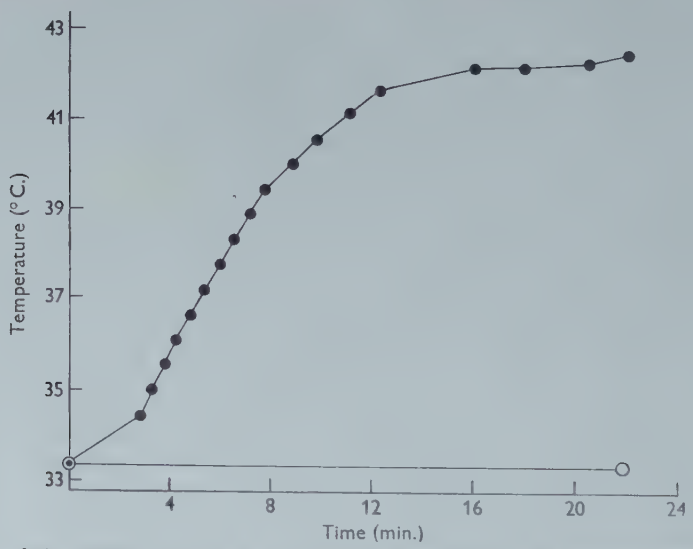

Fig. 2. Effect of the stilted posture on the internal temperature of *Opisthophthalmus latimanus* exposed to direct sunlight. Open circles, experimental animals in stilted posture; closed circles, control in normal resting stance.

A typical result of such an experiment is shown in Fig. 2. Both animals were initially in the shade, their abdominal temperatures were recorded: the screen was then removed and the temperatures of their bodies noted at intervals. As can be seen, the temperature of the control animal rose rapidly, while that of the scorpion held in a stilted position hardly rose above the minimal temperature calibration of the thermometer.

* It must be noted here that the initial temperature of each of the scorpions, 91–93° F. (33–34° C.) reflects, in the main, only the minimal reading on the thermometers used.

The question now arises as to how the stilted pose prevents the large increase in abdominal temperature found in animals held in the resting position. Three possibilities have been considered. The first is that the space provided between the body and substratum by the stilting allows evaporation to occur from the openings of the book-lungs, and that this cools the scorpion's body. The second is that the stilting merely lifts the body away from the substratum so that absorption of heat from this source is curtailed. Thirdly, it is possible that, by raising the abdomen, air currents are permitted to pass beneath and around the scorpion's body, thus cooling it to air temperature. These explanations are not, of course, mutually exclusive.

The first suggestion, namely, that stilting keeps the body temperature low by facilitating evaporation from the book-lungs, has some measure of support in the fact that in insects a high percentage of the cooling which is effected occurs by way of the respiratory openings (Wigglesworth, 1950). The point is, however, easily investigated in the scorpion and Fig. 3*a* shows the result of such an observation on *Opisthophthalmus latimanus*.

The book-lungs of the experimental animal were painted over with Samsonite to prevent evaporation, initially both experimental and control animals were shaded from direct sunlight and it can be seen that, when the shade was removed, the body temperature of the experimental, stilting animal hardly altered, although that of the control rose rapidly. Certainly any evaporation from the book-lungs contributes only slightly, if at all, to the cooling effect of the stance.

Such a conclusion, though not in keeping with that expected from an insect, is supported by the findings of Parry (1951) with model disks and locusts, namely, that cooling by evaporation is likely to be less important than heat loss by radiation and convection in determining the body temperature of terrestrial arthropods placed in direct sunlight.

The second suggestion, that stilting decreases heat conduction and radiation from the ground surface, is not so easily investigated. Parry (1951) reports that the temperature of a small black disk exposed to direct sunlight will fall by as much as 12° C. if it is moved to a height of 1 in. above the substratum. He considers that at such small distances from the ground the steepness of the 'temperature profile' is probably due to a rapid fall off of radiation effects from the substratum and that convectional losses are likely to be low. To test the importance of convectional loss it is necessary to eliminate as far as possible all 'forced convection' (Digby, 1955), that is, all air currents caused by factors other than the presence of the scorpion itself. This was done by covering the animal, its stand and the thermometer with a bell-jar; both experimental and control scorpions were then exposed to direct sunlight.

As can be seen from Fig. 3*b*, the temperature of the control animal again rose rapidly, but on this occasion, after a short lag, so also did that of the experimental animal. The glass cover was then removed from the latter while the control was shaded from the sun. The body temperature of both animals then fell quickly.

To check that the effect of the glass cover over the scorpion was to decrease the rate of cooling and not to increase the rate of heating by concentrating the sun's rays upon the animal, the simple test of lifting the cover an inch from the substratum was made. Under such conditions the temperature of the stilting scorpion remained nearly constant, as though the cover had been completely removed.

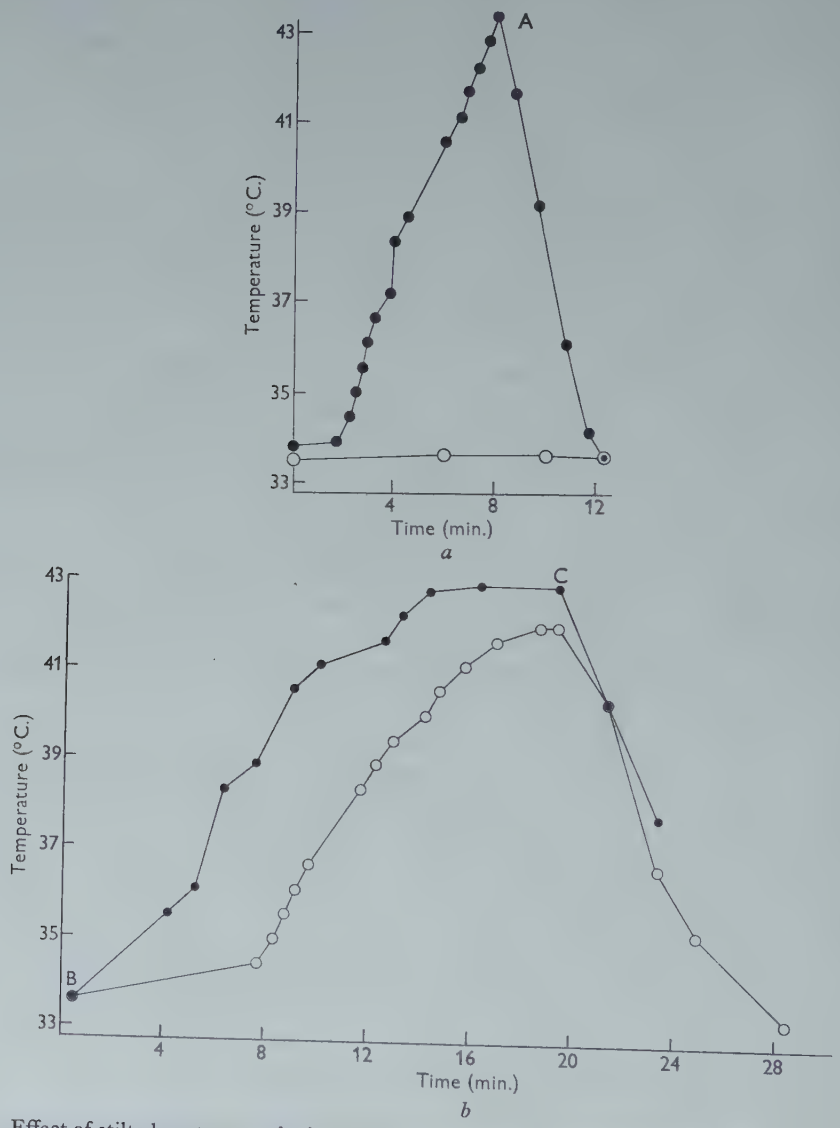

Fig. 3. Effect of stilted posture on the internal temperature of *Opisthophthalmus latimanus*. (*a*) comparison of normal animal in resting stance—closed circles; and experimental animal with book lungs sealed in stilted pose—open circles. Initially both animals shaded but exposed to direct sunlight at time o. At *A* both animals again shaded. (*b*) a continuation of the experiment slightly later. Both animals initially shaded. At *B* a glass bell jar was placed over the experimental animal and both exposed to sunlight. At *C* the cover was removed from the experimental animal and the control was shaded from direct sunlight.

These results indicate strongly that the temperature-controlling effect of the stilting behaviour is due mainly to an enhanced circulation of air around the scorpion. Since the above effects may all be imitated using recently killed animals held in suitable positions, there is no suggestion that the cooling phenomenon is due to any 'vital' activity of the animal. This incidentally supports Parry's assertion that in dealing with arthropods of a fair size, it is legitimate to work with models of reasonable size and shape.

These results with *Opisthophthalmus latimanus*, while not eliminating the possibility that stilting may lower heat uptake by radiation from the substratum and by natural convection, suggest that the exposure of the whole body surface to local air currents is a more effective mechanism of temperature control. It seems likely that this may be of general importance in the temperature regulation of other arthropods such as ants and certain tenebrionid beetles where patterns akin to stilting are known to occur. The precise importance of local air currents was not directly studied by Parry in his work with models. Digby (1955), who investigated the point with controlled conditions and live animals, reports that, with wind speeds of 20–30 cm./sec., the animal's temperature excess (i.e. the difference between that of the animal and the surrounding air) varies inversely with the square root of the wind speed. Below this wind-speed the natural convection of the animal itself became of more importance. As Digby points out, it is unfortunate that we know so little of wind speed and other microclimatic factors close to the ground.

Once it is clear that the adaptive significance of the stilting behaviour pattern is one of thermo-regulation, two further questions arise: first, where are the thermo-receptors situated and secondly, what are the circumstances in which the pattern is evoked in natural conditions?

THERMO-RECEPTORS

Nothing appears to be known of the location of thermo-receptors in scorpions, nor in fact in other arachnids. It appears desirable to point out initially that stilting is not simply a direct response to the temperature of the substratum, that is, the animal does not lift its belly away from the 'burning' ground. This can be shown by placing the scorpion on a copper plate with running cold water beneath and then directing a heating radiator on to the animal's body from above. Although the substratum on which the animal stands is very much cooler than the air above it, the scorpion will raise its abdomen into the warmer layer of air.

It can readily be shown that the stilting response to high temperature persists after the pectines have been removed; similarly, removal of the pectines does not alter the preferred temperature when the animals are studied in a gradient. Further operative procedures are impossible and the point was therefore examined by observing the response of scorpions to a red-hot cauterizing needle held near different parts of the body. These experiments showed that the poison bulb of the sting is very sensitive to a local heat source; the pedipalps are also sensitive though seemingly less so than the sting; the sensitivity of the legs is still less marked, while no evidence was found for any thermo-receptors upon the back.

It would thus appear that thermal exteroceptors are widely scattered over the scorpion's appendages and, therefore, any attempt to eliminate them experimentally is effectively precluded; thus it cannot be determined whether the stilting response is mediated by way of these thermal receptors. Moreover, it seems possible that a postural thermo-regulatory reaction, such as this, may well be controlled by central thermo-receptors, responding to general body temperature, rather than by thermal exteroceptors.

Some measure of support for this suggestion comes from a consideration of observations on a number of *O. latimanus* which were heated until they adopted extreme stilting poses. Then, with as little disturbance as possible, they were moved to cool dishes in conditions where controls showed no sign of stilting. Here four, of seven animals used, reverted to the stilting pose for at least 3 min. and only later relapsed to the normal resting posture. This indicates that when scorpions have been well heated they may sometimes stilt in a cool dish where exteroceptors would not be receiving stimulation from the environment.

STILTING IN RELATION TO LIFE IN NATURAL CONDITIONS

In attempting to answer the question 'When does *O. latimanus* stilt under natural conditions?' it is desirable to consider first some other aspects of the biology of this scorpion.

The animals live in burrows about 30 cm. deep. In the laboratory they have been allowed to burrow in the soil of glass-sided aquaria and in these burrows their activities may often be watched without disturbing the animals at all. Such observations indicate that scorpions in the laboratory spend many hours of daylight at the entrance of their burrows or 2 or 3 cm. down them. A limited number of observations were made in the field and these confirm the fact that *O. latimanus* is not confined to the depths of its burrow during the day. Excavation of the burrows by the scorpion normally occurs in the late afternoon or at night, both in the field and in the laboratory, while after sundown the scorpions in the laboratory terraria may leave their burrows completely and wander some distance from them. Whether this occurs in the field at night is uncertain owing to the difficulties of observation, but occasionally in the late afternoon an animal has been found away from its burrow.

It seems probable that this 'door-keeping' which occurs during the daylight hours is connected with feeding. Certainly a study of food fragments in and around their burrows shows that *O. latimanus* feeds upon insects such as grasshoppers and a common tenebrionid beetle which are active during the day. Further, a scorpion at the entrance to its burrow will grab viciously at a stick which is moved carefully and 'temptingly' towards it, while a partially immobilized grasshopper placed near the entrance of the burrow will be hastily dragged down by the tenant.

Using a temperature 'orgel' it has been established that the temperature preference of *O. latimanus* lies within the range 32–38° C. The soil temperature at the mouth of the burrows may rise as high as 70° C. So that it would seem that

O. latimanus must be able to tolerate temperatures considerably higher than its preferred one if it is to sit in its burrow mouth and catch prey which comes within grabbing distance. Theoretically the stilting pattern would be of considerable use in such circumstances; in practice it has very frequently been observed to occur in the laboratory terraria where the scorpions are door-keeping during the warm part of the day. Observations in the field are not possible as only the pedipalps of a door-keeping scorpion can be seen clearly.

BEHAVIOUR RESORTED TO WHEN STILTING BECOMES INADEQUATE

It has been found that the lethal temperature of *O. latimanus* lies roughly in the range 40–50° C. It appeared of interest to find out whether, if the temperature of its body approaches the lethal temperature, a scorpion will merely continue to stilt or whether some other pattern is evoked. The answer to this question involves a consideration of responses to directional light.

Many scorpions show a marked photonegative response to directional light. This is true of *Androctonus australis*, *Scorpio maurus* L. and *Buthus occitanus* (Am.) (Sergent, 1947). Of the species studied here it is also true of *Parabuthus planicauda*, but *Opisthophthalmus latimanus* and *O. nitidiceps* show a very striking photopositive reaction. Further, in agreement with the observations of Sergent upon the various European species mentioned above, *O. latimanus* shows well-marked thigmopausic behaviour. A combination of these two elementary behaviour patterns would serve to direct *O. latimanus* to the entrance of its burrow: so long as no other pattern interferes, the scorpion could be expected to remain at the mouth of its burrow, facing the light but not leaving the contact provided by the walls. As has been emphasized above, should a scorpion maintain this position upon a hot day the environmental temperature might well exceed the lethal temperature for several hours and the immediate problem is whether a rising temperature releases an escape reaction.

The problem was studied by placing individuals in long glass troughs whose floors were covered with soil. All the sides of the trough were blackened except one, through which there shone a light. The temperature of the trough could be changed as required. In such a piece of apparatus at room temperature, the scorpions show a marked preference for the end of the trough nearer the light source. As the temperature is raised the scorpions will stilt, but they remain oriented towards the light source. Then with a further increase in temperature there is a sudden reversal of the sign of the light response, the scorpion turns away from the light and moves rapidly to the other end of the trough. If the temperature is then allowed to fall, the scorpion presently reorients towards the light.

Often just before the change of photopositive to photonegative behaviour occurs, there are signs of a general activity: frequently these movements belong to no obvious pattern, sometimes the animal will abruptly, if ineffectively, begin to burrow. The exact interpretation of this latter is not at the moment clear: it might be regarded as an attempt to construct a burrow for protection, a mere effort to get

away from an unpleasant stimulus or a displacement activity arising from a conflict between opposing photopositive and photonegative drives.

Interpreted in terms of its normal life, these observations imply that as temperatures rise and general activity of the animal increases, the light reaction will not direct the animal in such a way as to cause it to leave the safety of its burrow, but rather its reversal of sign will result in the scorpion retreating into the deeper parts which, as rough measurements in the field have shown, may be more than 20° C. below the soil temperature outside. It would seem that this reversal of light behaviour is the basis of an escape from a potentially lethal position.

It is interesting to note that the protective behaviour pattern of this scorpion in relation to high temperature has two facets—a static postural behaviour pattern which permits a certain degree of regulation, followed by a dynamic locomotory pattern which allows the animal to evade the difficulty by leaving the potentially lethal environment.

During the course of this investigation one of us (A. J. A.) held a bursary granted by the South African Council for Scientific and Industrial Research, to whom our thanks are due.

SUMMARY

1. Behaviour termed 'stilting' is described for the scorpion, *Opisthophthalmus latimanus*. In this pattern the legs are straightened, lifting the body clear of the substratum.

2. Evidence is submitted that it is not concerned with allowing greater respiratory exchange.

3. Stilting is generally elicited in response to a rise in environmental temperature above 18° C. and is invariably found at temperatures above 28° C.

4. A comparison using scorpions held in the stilted and normal resting stance, shows that, when the environmental temperature rises sharply, the body temperature of the resting animal rises rapidly, while that of the stilting animal is almost unchanged. The mechanism of this effect is shown to be due largely to the increased circulation of air around the animal which is permitted by the stilting.

5. From observations of behaviour in both the laboratory and the field, it appears probable that the stilting pattern is shown by *O. latimanus* during the hot hours of the day when the scorpion waits in the entrance of its burrow to catch prey.

6. Laboratory observations indicate that when the temperature becomes so high that stilting has no longer any protective value, a photopositive reaction, which would keep the scorpion at the entrance of its burrow, changes to a photonegative one and the animal can retreat into the cool depths of its burrow.

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THE OXYGEN DISSOCIATION AND ALKALINE DENATURATION OF HAEMOGLOBINS FROM TWO SPECIES OF EARTHWORM

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INTRODUCTION

Although it has been clear since the work of Fox (1940) and Johnson (1942) that the haemoglobin of *Lumbricus* can take part in oxygen transport in the normal animal, the oxygen dissociation curves of this haemoglobin have not been available. The present paper provides oxygen dissociation curves for haemoglobin from two species of earthworm, *Lumbricus terrestris* (Linnaeus) and *Allolobophora terrestris* (Savigny) forma *longa* (Ude). Temperature has a direct effect on the oxygen dissociation and for this reason the haemoglobin dissociation was recorded at two temperatures approaching the upper and lower limits of the normal earthworm habitat. The results show that the haemoglobins of the two species have different properties and these differences are further confirmed by studies on the rate of alkaline denaturation of the respective haemoglobin solutions.

METHOD

Preparation of blood

The earthworms used in these experiments were *Lumbricus terrestris* (Linnaeus) and *Allolobophora terrestris* (Savigny) forma *longa* (Ude), and they were selected because of their size and availability.

The animals were anaesthetized for a few seconds over chloroform and a lateral slit was made in the body wall in the region of the gizzard; the flaps were pinned down and the coelomic fluid removed by filter-paper. A cut was then made across the ventral blood vessel and the blood collected by insertion of a finely drawn Pasteur pipette. A single *Lumbricus* yielded approximately 30–50 mm.^{3} of blood, while *Allolobophora* yielded less in proportion to size. On removal the blood was immediately frozen and stored over dry ice, and under these conditions remained stable for several days. Each experiment was carried out on a sample of pooled blood obtained on the same day from approximately thirty-six animals. The worms were collected from similar localities at the same time.

When required the blood was brought to the experimental temperature and 0·05 ml. of 3 M phosphate buffer added per ml. of blood (Green, 1933) to give a final pH of 7·3 and a phosphate concentration of 0·15 M. A drop of octyl alcohol was

then added to prevent foaming when the blood was later subjected to low pressures. Finally the blood was centrifuged to remove any particulate matter present.

Determination of the oxygen dissociation curves

The method used had to satisfy the conditions that: (i) only small quantities of blood were required; and (ii) it was applicable to haemoglobin concentrations approximating to those in the intact animal. This latter is necessary since many haemoglobins are known that are only stable at normal physiological concentrations, and furthermore the oxygen affinity of a dilute solution is not necessarily the same as that of haemoglobin *in vivo* (Hill & Wolvekamp, 1936).

The method chosen was based on that of Redfield (1930), the blood being equilibrated at various partial pressures of oxygen and the percentage of dissociation determined spectrophotometrically.

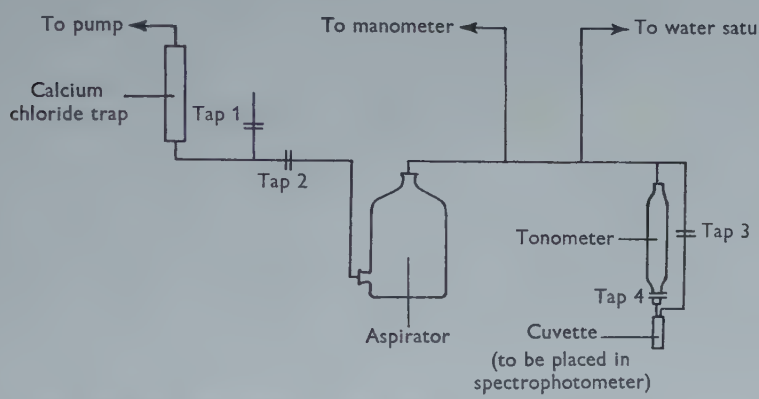

Fig. 1. Block diagram of apparatus used to determine the oxygen dissociation of earthworm haemoglobin. For further details see the text.

The apparatus chosen for equilibration of the blood consisted of a 100 ml. cylindrical separating funnel which served as a tonometer. A range of oxygen pressures was obtained by evacuating the air of the tonometer to the required degree. The tonometer was connected in series to an Edwards rotary vacuum pump through a 5 l. aspirator and a calcium chloride trap (Fig. 1). Between the aspirator and tonometer were two side connexions, one leading to a mercury manometer, the other leading to a 100 ml. vessel containing water so placed as to keep the system saturated with water vapour, thus minimizing evaporation from the blood. Between the calcium chloride trap and the aspirator was a tap 2, which separated the system from the pump during equilibration. Air could be admitted through tap 1.

The water-vapour pressure was constantly checked by a wet-bulb thermometer placed in the aspirator. In general, on initial pumping the vapour pressure decreased slightly, but when the pump was cut off and tap 2 closed the system rapidly became saturated with water vapour. The internal pressure of the tonometer was calculated

by subtracting the recorded manometric pressure and the appropriate vapour pressure at that temperature from the barometric pressure. The partial pressure of oxygen then was calculated by multiplying this pressure by the fraction of oxygen present in dry air.

The optical density of the blood was measured at 660 mμ (Roddie, Shepherd & Whelan, 1956) in a Unicam S.P. 600 spectrophotometer, and for this purpose a special cuvette was made from selected pieces of microscope slide, the edges being sealed with Apiezon wax. The cuvette had a thickness of 1·1 mm. and held approximately 0·55 ml. of blood. It was fitted with two tubes, a delivery tube terminating at the bottom of the cuvette (to avoid formation of air locks) and an outlet tube at the top (Fig. 2). The delivery tube was connected through tap 4 to the tonometer while the outlet tube was connected by pressure tubing through tap 3 to the vacuum line.

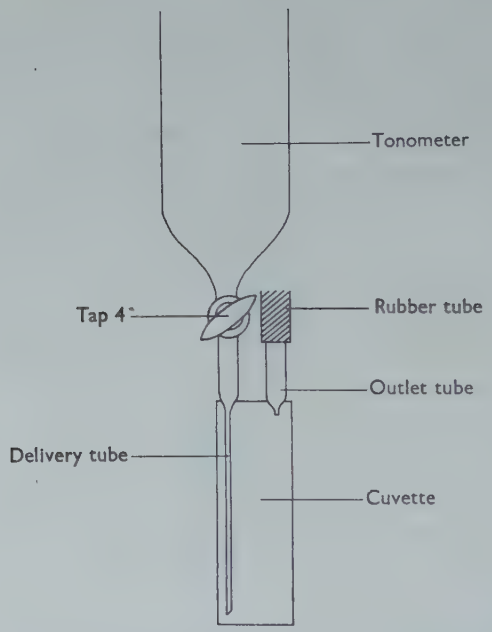

Fig. 2. Tonometer and cuvette. The cuvette was made from cut pieces of microscope slides. Note that the delivery tube ends near the base of the cuvette; this prevents the formation of airlocks when the blood is transferred from tonometer to the cuvette.

In operation the tonometer was detached from the vacuum line and with tap 4 closed the blood sample was introduced. The tonometer was then reconnected to the vacuum line and the pressure reduced to the required level. The blood was then equilibrated in the tonometer at each partial pressure for 15 min. During this time the tonometer was gently agitated by hand in the near-vertical position with tap 4 closed, thus minimizing loss of solution by spreading on the walls. When equilibration was complete tap 4 was opened and the blood flowed into the cuvette.

The cuvette with its attached tubes was then placed in the Unicam cell holder and held in position by a Perspex holder which allowed the passage of tubes to the cuvette. The holder with cuvette was then placed in the spectrophotometer and the optical density measured, in a series of determinations in which the partial pressure of oxygen was progressively reduced.

After determination of the optical density the tonometer and cuvette were removed from the spectrophotometer and held in a horizontal position with the delivery tube on the lower side of the cuvette. The blood was then returned to the tonometer by slightly raising the pressure of the system with tap 4 closed and tap 3 open, followed by lowering the pressure with tap 4 open and tap 3 closed. The blood was then ready for re-equilibration at another partial pressure of oxygen.

During equilibration, provided that frothing is controlled, little or no denaturation of the protein occurs. The final measurements for the completely reduced solution were obtained by the addition of a trace of sodium dithionate.

The percentage saturation of the haemoglobin, at any given partial pressure, was calculated from the recorded optical density at 660 mμ by linear interpolation between the values for the fully oxygenated and for the fully deoxygenated condition.

RESULTS

The experiments on the dissociation of the blood were carried out in constant temperature rooms at 7° and 20° C. Each experiment was performed on a pooled blood sample adjusted to pH 7·3 as described earlier.

Fig. 3 shows the points of a dissociation curve for the blood of *Lumbricus terrestris* at these two temperatures. Each curve shows the points from two different samples of blood collected some 2 weeks apart, and as can be seen there is good reproducibility. The points for the lower temperature (7° C.) lie on a less sigmoid curve and to the left of those for the higher temperature (20° C.). The partial pressure of oxygen at which the blood is 50 % saturated is equivalent to 2 mm. Hg at 7° C. and 8 mm. Hg at 20° C., and the partial pressure of oxygen at which the blood is 95 % saturated is 9 mm. Hg at 7° C. and 22·5 mm. Hg at 20° C.

Fig. 4 shows a similar series of points for the blood of *Allolobophora terrestris*. Here too the points at the lower temperature lie on a less sigmoid curve and to the left of those at the higher temperature. The partial pressure of oxygen at which the blood is 50 % saturated is 0·7 mm. Hg at 7° C. and 6 mm. Hg at 20° C. and the blood is 95 % saturated at 3·75 mm. Hg at 7° C. and 17·5 mm. Hg at 20° C.

These results are in general agreement with those obtained by Barcroft & King (1909) and Macela & Seliskar (1925) for the blood of man, tortoise, frog and *Planorbis*. These authors found that when all other factors, such as ionic strength, pH, and protein concentration were kept constant, a change in temperature brought about a shift in the dissociation curves. At lower temperatures the curves became less sigmoid and moved to the left of the graph, i.e. the haemoglobin had a greater oxygen affinity at lower temperatures.

The figures also show that there is a difference between the dissociation of the blood of *Lumbricus* and *Allolobophora*, and this difference is more clearly seen in

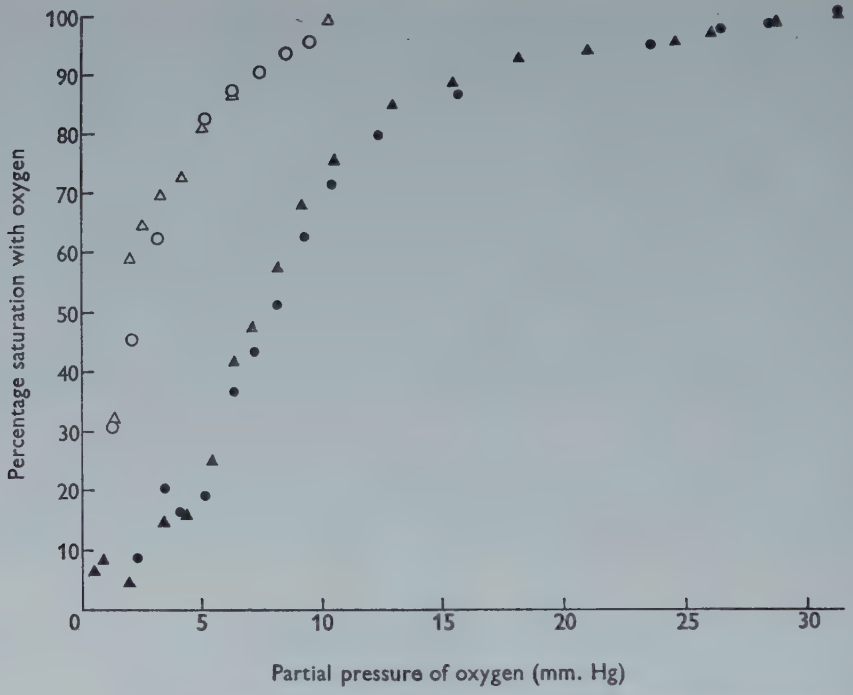

Fig. 3. Oxygen dissociation curves of haemoglobin from *Lumbricus terrestris*. The open symbols refer to 7° C., the black symbols to 20° C.

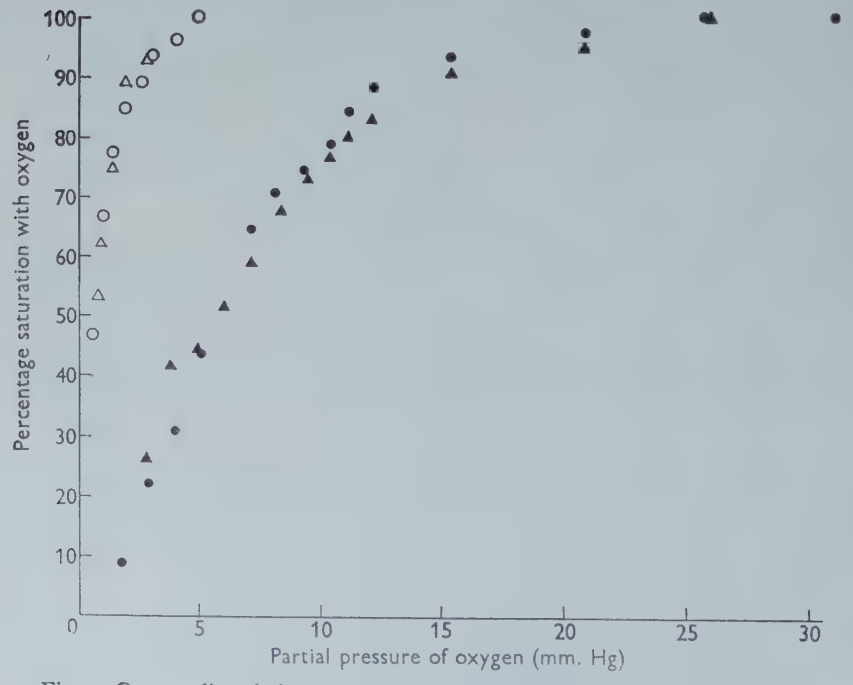

Fig. 4. Oxygen dissociation curves of haemoglobin from *Allolobophora terrestris*. The open symbols refer to 7° C., the black symbols to 20° C.

Fig. 5 where the four curves are plotted together. The curves for *Allolobophora* blood at a given temperature lie to the left of those for *Lumbricus* at the same temperature. This indicates that the haemoglobin of *Allolobophora* differs from that of *Lumbricus* and has a higher oxygen affinity.

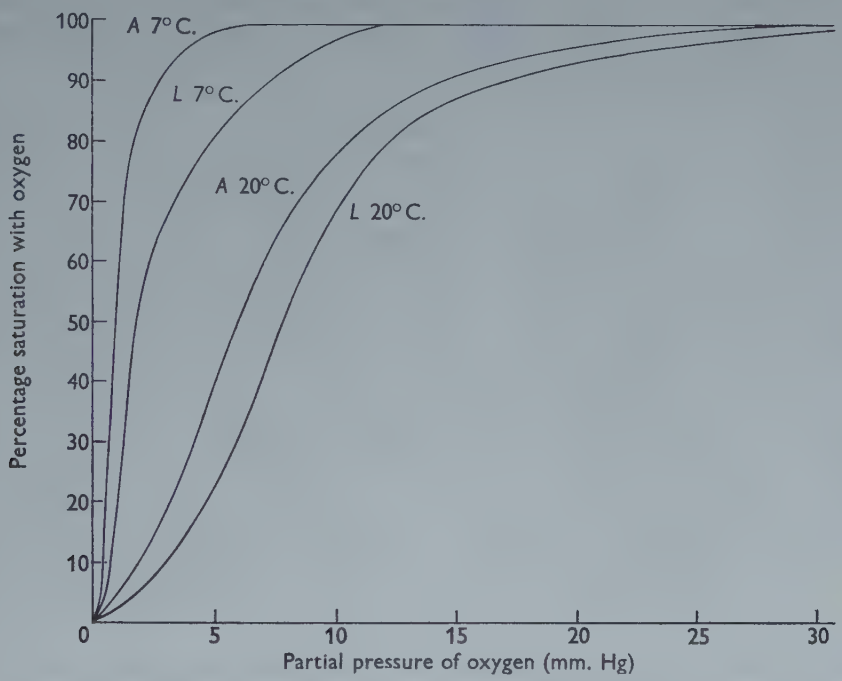

Fig. 5. Superimposed oxygen dissociation curves of haemoglobin from *Lumbricus* and *Allolobophora*. The curves for *Allolobophora* lie to the left of those for *Lumbricus* at the corresponding temperatures. *A* refers to *Allolobophora*, *L* to *Lumbricus*.

Alkaline denaturation of earthworm haemoglobin

Another method by which differences between haemoglobins have been previously demonstrated makes use of the difference in the rate of alkaline denaturation (Brinkman & Jonxis, 1937; Ramsey, 1941). Alkaline denaturation rates were therefore studied on blood samples from these two species of earthworm.

In these experiments the oxyhaemoglobin is converted under controlled conditions to alkaline globin haemochromogen (Lemberg & Legge, 1949) and the reaction is followed spectrophotometrically. The earthworm blood was diluted with distilled water to bring it to a concentration of 0·12 g. haemoglobin/100 ml. of solution. 2·5 ml. of the diluted blood was placed in a 1 cm. cuvette and the cuvette placed in a constant temperature holder at 25° C. The holder was inserted into a Unicam S.P. 600 spectrophotometer and 0·5 ml. of glycine buffer (Vogel, 1945) was added to the cuvette to bring the final pH to 12·7. The extinction coefficient at 541 mμ. was measured at intervals of 30–60 sec. until the readings became constant. From these results the percentages of unchanged oxyhaemoglobin remaining in the solution could be calculated, and were then plotted against time. Fig. 6 shows representative

curves of blood from the two species. It will be seen that the blood of *Allolobophora* denatures much more rapidly than does that from *Lumbricus*, *Allolobophora* blood being denatured in just under 6 min. as compared with 23 min. for *Lumbricus*. We have found that three other species of earthworm have specific denaturation times: namely, *Lumbricus rubellus* 2·5 min., *Eisenia foetida* 5 min., and *Lumbricus festivus*, 11·5 min. Sufficient numbers of these other species were not available for determination of the oxygen dissociation curves.

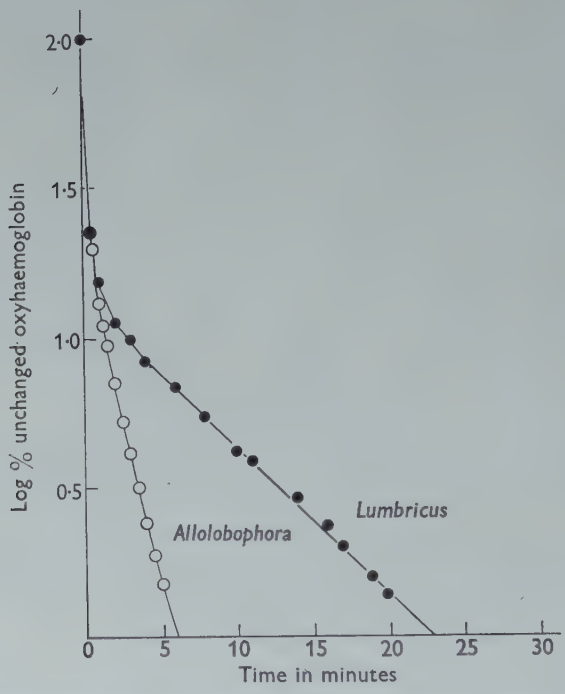

Fig. 6. Rate of alkaline denaturation of haemoglobin solutions from *Lumbricus* and *Allolobophora*. *Allolobophora* haemoglobin denatures more rapidly than does that of *Lumbricus*.

DISCUSSION

The oxygen dissociation curves of earthworm haemoglobin have not previously been determined. The curves described in the present paper agree fairly closely with those recorded for other annelids, though in some cases other workers have used diluted bloods and worked at different temperatures, all of which tend to alter the position of the curves. Some figures that can be roughly compared are as follows. The partial pressure of oxygen necessary for 50% saturation is 2 mm. Hg for *Lumbricus*, 0·7 mm. Hg for *Allolobophora*, 1·5 mm. Hg for *Arenicola*, 0·5mm. Hg for *Tubifex* (Fox, 1945), 5·5 mm. Hg for *Nephthys* (Jones, 1955) and less than 1 mm. Hg for *Alma* (Beadle, 1957). These values are generally appreciably lower than those recorded for vertebrate haemoglobins (Prosser, Bishop, Brown, Jahn & Wulff, 1950).

It is interesting to consider whether the difference in the dissociation curves of *Lumbricus* and *Allolobophora* are related to differences in the physiological behaviour of the two species.

In general, earthworms inhabit the top layers of the soil but we noticed in digging for worms that as the summer drew on *Allolobophora* became more difficult to find, while *Lumbricus* remained quite common. Evans & Guild (1947) showed that during the summer *Allolobophora terrestris* goes into aestivation, curling up into a tight ball 1–2 ft. below the surface of the ground. Baweja (1939) stated that the soil temperature follows the atmospheric temperature down to about 8 in. below the surface, while the temperature is roughly constant throughout summer and winter at a depth of 3 ft. It is possible that the behavioural difference could be due in part to respiratory difficulties, but the difference in the curves for the blood pigments is in fact greater at a lower temperature than at a higher one. Consequently no correlation can be seen between the respiratory pigment properties and the physiological behaviour of the earthworms. It seems possible that the summer aestivation is affected by some other factor such as the availability of water; *Allolobophora* has a different nephridial pattern from that of *Lumbricus* (Goodrich, 1945).

In many animals such as the sheep, goat and cow, the haemoglobin of the foetus has a dissociation curve lying to the left of that for the adult. Brinkman & Jonxis (1937) showed that the foetal haemoglobin was less resistant than adult haemoglobin to alkaline denaturation. Similarly, in earthworms the dissociation curves of *Allolobophora* lie to the left of those of *Lumbricus*, and the haemoglobin is less resistant than *Lumbricus* haemoglobin to alkaline denaturation.

It is not clear whether the alkaline resistance of haemoglobin is directly related to the oxygen affinity of the haemoglobin especially when considered intergenerically. What is reasonably clear is that it does provide a method of distinguishing between types of haemoglobins and in this it has shown that the *Allolobophora* and *Lumbricus* haemoglobins differ.

We hope at a later date to examine the paramagnetic resonance of haemoglobin crystals from *Lumbricus* and *Allolobophora* blood in the hope that this too will throw further light on the different properties of the haemoglobins (Bennett, Gibson, Ingram, Haughton, Kerkut & Munday, 1957).

SUMMARY

1. A method is described for determination of the oxygen dissociation curves of the blood of two species of earthworm, *Lumbricus terrestris* and *Allolobophora terrestris*.

2. The following values have been obtained for the partial pressures of oxygen (mm. Hg) required to saturate the blood:

	50% saturation		95% saturation	
	7° C.	20° C.	7° C.	20° C.
Lumbricus	2	8	9	22·5
Allolobophora	0·7	6	3·75	17·5

3. Times for alkaline denaturation at pH 12·7 have been found as follows: *Lumbricus terrestris* 23 min., *L. rubellus* 2·5 min., *L. festivus* 11·5 min., *Allolobophora terrestris* 6 min., *Eisenia foetida* 5 min.

4. These results indicate definite differences in the properties of the haemoglobin of the species examined.

This investigation has been supported by a grant from the Medical Research Council to one of us (K.A.M.).

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THE CO-ORDINATION OF THE RESPONSES OF *CERIANTHUS* (COELENTERATA)

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(*Received* 30 *October* 1957)

Cerianthus membranaceus, as commonly available at Naples, is a brownish polyp which can extend to 15–20 cm. and contract to 5–7 cm. The numerous tentacles are less contractile, being usually 3–5 cm. long. The gross anatomical structure is well described by Hyman (1940). The polyp normally lives in a soft tube, which it secretes, and has no foot or sphincter muscle. Lacking the diversity of muscle groups of a typical sea anemone its movements are very restricted. In contrast to other coelenterate polyps the Ceriantharia have a substantial ectodermal musculature of the column, which is responsible for the vigorous withdrawal of the animal into its tube. There are also ectodermal radial fibres on the disk. Endodermal muscles are relatively insignificant and play no part in the responses considered here. In common with all other coelenterate polyps *Cerianthus* has two kinds of rapid response to external changes: the feeding movements and the protective retraction. In *Cerianthus* the two are aroused by different stimuli, and in part they are effected by different muscles. The present study is an analysis of some of the physiological properties of the pathways which co-ordinate these responses.

The histology of the nervous system of *Cerianthus* is a controversial topic and, apart from sensory cells, structures with the appearance and staining properties of neurones have not so far been described with certainty. But this is not for lack of a search. A number of histologists have studied *Cerianthus* during the course of the last century, and have used most of the currently available techniques for the selective staining of nerve cells. This work has been recently summarized by Torelli (1938, 1952), who has added her own observations. She comes to the conclusion that the only identifiable nerve elements are sensory cells with branched fibres connecting directly with the ectodermal muscles. This arrangement is exceptional among coelenterates, and moreover there is no indication of how it could co-ordinate the observed movements. On the other hand, the results to be described suggest that a through-conducting network of large axons is present. However, the results of stimulation cannot as yet be correlated with a histological nerve net such as is usually presumed to account for at least some of the responses of coelenterates. Even ganglion cells, or their nuclei, have not been found with certainty. Therefore in the present study, which is solely physiological, we cannot consider axons or synapses but only the excitability and actions of the conducting pathways.

The responses of the Ceriantharia have been largely neglected, perhaps because

they are a small aberrant group, though common as aquarium exhibits. Reference will be made to the work of Moore (1927*a*, *b*) on the responses to light and to gravity. That a symmetrical twitch follows a single electrical shock was noted by Ross (1957), but the methods of electrical stimulation as introduced by Pantin (1935) for the study of coelenterate responses have not so far been applied.

These methods have one serious disadvantage in that the resulting analysis does not give a balanced picture of the co-ordination of the animal. Electrical stimulation evokes the protective retraction of coelenterate polyps. However, other examples of co-ordinated activity are continually observed, particularly in feeding, creeping, burrowing and peristaltic movements. The physical properties of the through-conducting systems, as deduced from electrical stimulation experiments, are now known for several species of anemones and corals, but the other activities are much less amenable to present experimental techniques. This disadvantage is encountered also in the present study of *Cerianthus*: the protective retraction can be readily analysed, but the pathways which co-ordinate the feeding response and the slower movements remain unknown.

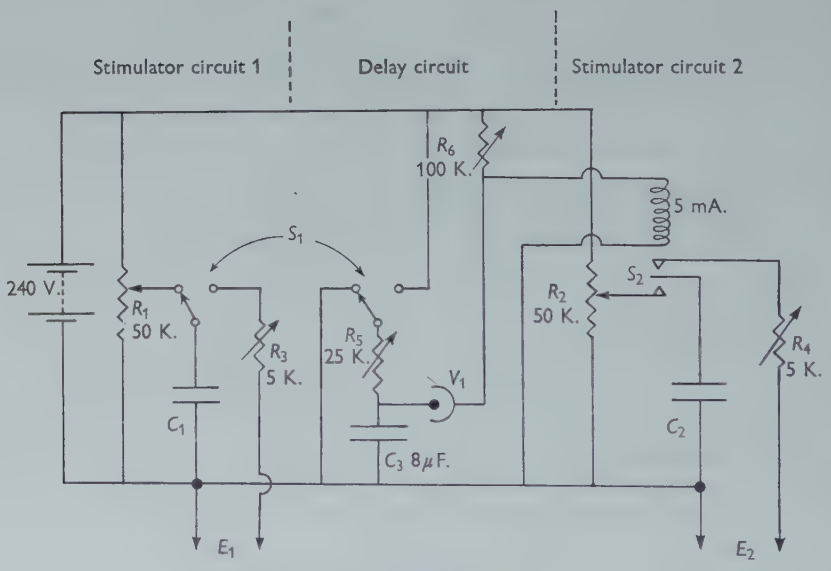

Fig. 1. For explanation see text.

METHODS

The most convenient stimulus for the study of coelenterate through-conducting systems is the condenser discharge. The effects can be related by theoretical equations or by direct experimental comparison to those produced by pulses of constant current. The arrangement used in the present work is shown in Fig. 1. It consists essentially of two stimulator circuits linked together by a delay mechanism.

In the stimulator circuits the condensers C_1 and C_2 (variable by substitution from

0·01 to 32 μF.) are charged from the strength controls R_1 and R_2 and are discharged through the stimulating electrodes E_1 and E_2. In series with the electrodes are the variable resistors R_3 and R_4, by means of which the total resistance of each discharge circuit is adjusted. In the experiments the total external resistance was usually 3000 Ω. The first stimulator circuit is operated by a snap-action double-pole-double-throw switch, S_1, which also initiates the operation of the delay circuit. In the delay circuit the rate of charging of the condenser C_3 is controlled by the variable resistor R_5; when the voltage across C_3 reaches the striking voltage of the neon V_1 (VS 110) the condenser is discharged through the coil of a relay which operates the contacts S_2 of the second stimulator circuit. The relay is aided by a trickle current adjusted by the variable resistor R_6.

This instrument, which is portable and weighs less than 1 kg., provides two stimuli of independently variable strength and duration and separated by a controlled interval of time. By adjustment of R_5 and C_3 a continuous sequence of stimuli at a frequency of about 1/sec. can be produced at the electrodes E_2.

The accuracy depends upon the linearity of the potentiometers and the precision of the condensers; by selection of ordinary components a precision of 5 % can be easily achieved. The control of the delay interval by R_5 is calibrated by means of an oscilloscope. Reproducibility is limited by contamination of the contacts, by changes in V_1 and by the friction of the relay. Repeated tests gave a standard deviation of about 5 % of the mean delay interval.

Non-polarizable $Zn/ZnSO_4$ electrodes, sealed into glass tubes by sea-water agar, were used for all experiments in which stimulus strength was measured.

Experiments were conducted at a temperature of 21–23° C.

RESPONSES TO STIMULATION

Cerianthus has two sets of tentacles, an inner and an outer group. In specimens of about 10 cm. expended length the inner tentacles are 1–1·5 cm. long and form a dense cluster round the mouth; the outer tentacles are 4–6 cm. long and are arranged in several rows round the margin of the disk. The tentacles of the inner group are continually making small spontaneous bending and waving movements which are intensified following light mechanical stimulation; those of the outer set are usually quite still unless disturbed. Both groups show the typical dual response to be described, but the following observations were made primarily on the tentacles of the outer row, which, being of greater size, are more easily studied.

When a tentacle is touched with a clean rounded glass rod no response follows. This agrees with von Uexküll's (1909) finding that chemical stimulation is necessary to elicit movement from *Anemonia* tentacles. A touch with the rod moistened with saliva produces a movement. Typically the tentacle contracts on the side touched and tends to curl round the rod. It shortens a little on both sides of the point of stimulation and swings towards the mouth by a movement at its base. This seems to be effected by longitudinal muscle fibres present in the tentacle itself and continuing on to the disk. A stronger mechanical stimulus alone, e.g. a gentle pinch,

evokes the above movements and, in addition, tentacles on either side of the one stimulated also swing towards the mouth. At the same time the edge of the disk is raised locally in the region stimulated and this movement helps to tilt the tentacles inwards. The muscles responsible for the edge-raising movement have not been identified. The movements spread round the disk to a greater extent as the irritation is continued. When one outer or inner tentacle is pinched with forceps all the outer tentacles at once respond with a co-ordinated inward movement, particularly in an animal which has been allowed to rest quietly for about an hour. The movement is propagated in both directions round the disk with a velocity of 2–4 cm./sec.

Besides the feeding movements spontaneous changes are continually going on at a much slower rate so that the outer tentacles change their positions with respect to the rest of the animal. In normal animals in an aquarium the innermost ring of the outer tentacles is sometimes raised vertically over the disk, with other tentacles partly raised, and at other times all the outer tentacles project at right angles to the column.

A single condenser discharge above threshold applied to any part of the tentacles or column initiates an immediate symmetrical twitch of the longitudinal muscle of the column. The extent of the contraction is independent of the strength of the stimulus, provided that it exceeds threshold. A single shock below threshold gives rise to a local bending of the tentacle at the point stimulated, together with a local shortening of the tentacle and an inward swing exactly as in the feeding response. Sometimes several such subthreshold shocks at intervals of about 1 sec. are required before the whole tentacle moves. With about ten shocks, at 1 per sec., neighbouring tentacles begin to respond also.. Such effects are found with the condenser charged to two-thirds of the voltage required for threshold stimulation of the through-conducting system.

Although this difference in threshold suggests two pathways of propagated excitation in the tentacles the observations as they stand do not necessarily indicate two conducting pathways. The through-conducting system cannot be excited by a stimulus to the tentacle without an accompanying local movement. Without other evidence the local movement could be merely an excitation of a smaller proportion of the units of a conducting system composed of many units. However, a stimulus applied to the column initiates an impulse that invades the tentacles antidromically and then they give only a slight twitch. This observation appears to confirm the view that the slower movements of the tentacles are co-ordinated by a physiological pathway that is independent of the through-conducting system. In addition we may tentatively suggest that at the input from the sensory cells to the through-conducting pathway there is a physiologically polarized junction which prevents the initiation of feeding movements by an antidromic impulse.

THE STRENGTH-DURATION RELATIONSHIP

That the threshold strength increases as the stimulus duration is reduced is an observation common to all excitable tissue, so the existence of a strength-duration relationship is not evidence that nerve axons are being excited. Other mechanisms,

such as that of *Vorticella* stalk, show the familiar curve (Lapique, 1926). Again, an absolute measure of the stimulus duration at a threshold of twice the minimum strength does not reveal the nature of the excitable process though it indicates its approximate time scale. I hoped that the excitability of *Cerianthus* could be defined more precisely if the observed strength-duration relationship proved to be a close approximation over a large range to the theoretical curve worked out by Hill (1936). However, Hill's theory does not apply specifically to nerves or to muscles; it only assumes certain formal quantitative relations. The form of the strength-duration curve could indicate how closely these relations account for the excitatory process and it might show to what extent *Cerianthus* resembles other coelenterates.

The stimuli, which were electrically negative condenser discharges of various time constants, were applied through non-polarizable $Zn/ZnSO_4$/sea-water agar electrodes in glass tubes drawn to an orifice of diameter 0·5 mm. For each series of measurements the total external resistance was adjusted to 3000 ohms with a high-resistance meter, a procedure shown to be adequate because the measuring current was the same in either direction and did not decay with time. The body of the animal and the sea water covering it were maintained at earth potential by a relatively large electrode. The small electrode tip was pressed against the region to be stimulated and held gently in place as the preparation moved. The variations following small movements were not large and an average over six to ten measurements was found for each type of stimulus.

Typical results from the stimulation of *Cerianthus* column are shown in Fig. 2(*a*), where they may be compared with the results (*c*) from the column of Naples specimens of *Calliactis*. These results from *Cerianthus* do not fit Hill's theoretical curve, which is drawn as a smooth line (*b*), whereas those for *Calliactis* are in reasonable agreement with the theory. Furthermore, the *Cerianthus* results are displaced well to the left of those for *Calliactis*. In Fig. 2 (*a*) which appears typical, the time constant RC at twice the rheobase is $1{\cdot}4 \times 10^{-4}$ sec. This is not the same as Lapique's chronaxie which relates to constant current pulses and is theoretically $0{\cdot}35\ RC = 5 \times 10^{-5}$ sec. The theoretical curve (*b*) has larger corresponding values; $2{\cdot}2 \times 10^{-4}$ and 8×10^{-5} sec. The theoretical curves all have the same shape; the constants determine only their position on the paper. For *Calliactis* (*c*) the time constant at twice rheobase is $4{\cdot}0 \times 10^{-3}$ sec.; Lapique's chronaxie is $1{\cdot}4 \times 10^{-3}$ sec. These constants were obtained by the method of curve-fitting described by Hill (1936).

The results are substantially the same wherever the stimulating electrode is applied on the surface from tentacle tip to aboral end. Fig. 3(*a*) shows results from stimulation of a tentacle. As in Fig. 2(*a*) they do not agree well with the theoretical curve. Chronaxie values ($0{\cdot}35\,RC$) ranging from 4×10^{-5} to 9×10^{-5} were found at different points on the surfaces of eight specimens, but no particular significance has emerged from these variations. The shape of the strength-duration curve differed slightly in some of the positions studied; for example, in Fig. 3 the observations indicated by crosses agree well with the theoretical curve (*c*).

The chronaxie for *Calliactis* is somewhat shorter than the figure of $2\text{–}4 \times 10^{-3}$ sec.

previously given by Pantin (1935); the chronaxie for *Cerianthus* is surprisingly short. A partial explanation of this curious result is to be found in the effective size of the electrode. Grundfest (1932) found that decrease of the electrode size is accompanied by a reduction of the chronaxie. With a capillary electrode 20–30 μ diameter on frog muscle fibres he obtained values 0·05 times normal, and for nerve 0·4 times normal. Pantin used large electrodes in pairs with the sea water not earthed, thereby producing effectively larger electrodes. In addition, in *Calliactis* a thick layer of mesogloea intervenes between the electrodes and the excitable

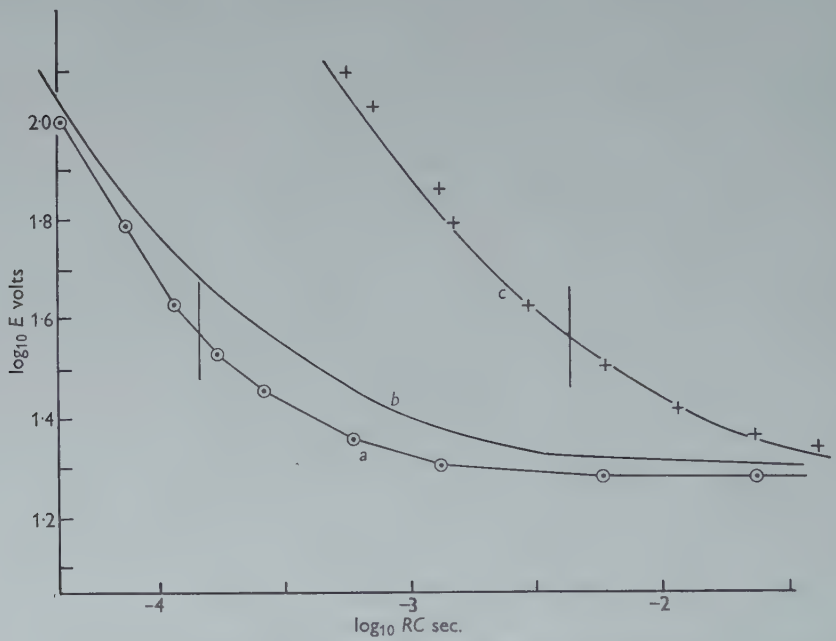

Fig. 2. The relation between intensity and time constant for the least stimulus required to excite. (*a*) Experimental results on *Cerianthus* column, with lines connecting the points; (*b*) curve of the theoretical equation $\log E = \log E_0 + [1/(RC/K - 1)] \log RC/K$ (Hill, 1936), with $K = 1{\cdot}0 \times 10^{-4}$, E_0 is the rheobase; (*c*) curve of Hill's equation, $K = 2{\cdot}5 \times 10^{-3}$, with new experimental results on *Calliactis* column. The vertical lines show the points at twice the experimental rheobase strength at (*a*) $1{\cdot}4 \times 10^{-4}$ sec. and (*c*) $4{\cdot}0 \times 10^{-3}$ sec. Each experimental point is the mean of six observations.

endoderm and this increases the effective electrode size since the current is more diffusely spread further from the electrode. In *Cerianthus*, the excitable tissue is ectodermal and is much nearer the electrode as here applied. The size and position of the electrodes do not account completely for the difference between the two species. Chronaxie measurements have also been made with the same electrodes on a variety of anemones, e.g. *Cereactis*, *Metridium*, in which the mesogloea is thin. The values so obtained agree with those from *Calliactis*. The mechanism of excitation peculiar to *Cerianthus* remains obscure; we are entitled only to draw the conclusion that it differs in time-scale from the corresponding mechanism of anemones.

These results at least show how difficult it is to identify the excitable tissue by indirect means and gross stimulation. The strength-duration curve fails as an aid in the identification of the excitability as nervous or muscular. But this need not always be the case; a composite curve with two chronaxies, as for some frog muscles, would be better evidence of two separately excitable tissues.

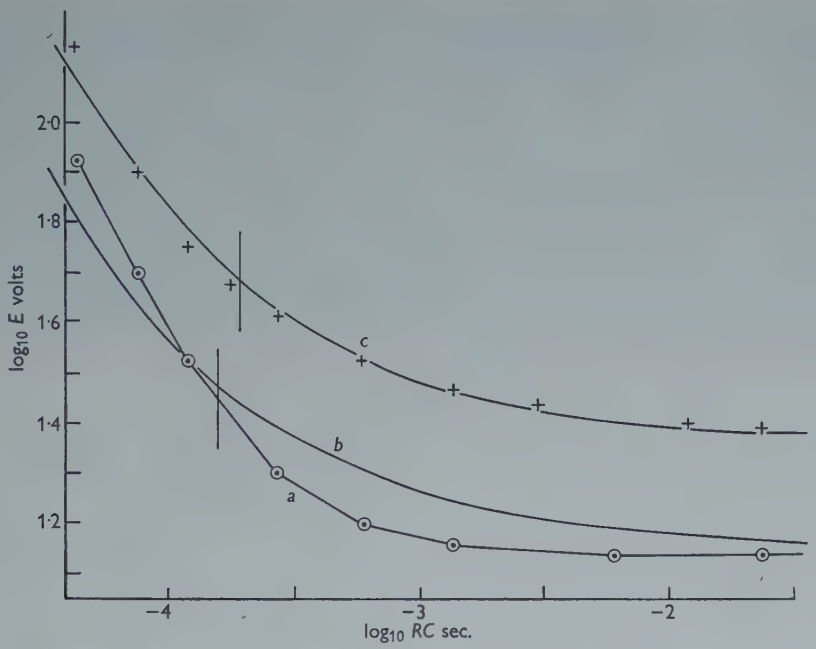

Fig. 3. The relation between intensity and time constant for the least stimulus required to excite. (*a*) Experimental results on *Cerianthus* tentacle, with lines connecting the points; (*b*) curve of Hill's equation (see Fig. 2) with $K = 1{\cdot}25 \times 10^{-4}$; (*c*) curve of Hill's equation with $K = 1{\cdot}6 \times 10^{-4}$ and experimental results on *Cerianthus* column. Note that in this example (*c*), in contrast with the results in Fig. 2(*a*), the results from the column agree with the theoretical expectation. As in Fig. 2(*a*) the results from the tentacle 3(*a*) follow a more inflected curve than the theory predicts. The vertical lines show the points of twice the experimental rheobase strength. Each experimental point is the mean of six observations.

THE REFRACTORY PERIOD

The refractory period was measured by applying a second shock of sufficient strength to excite at various intervals after an initial stimulus. The total contraction height is greater if the second stimulus produces a second impulse. The difference in the two contraction heights readily indicates whether the second stimulus has successfully excited the conducting system. The time constant of each stimulus was $1{\cdot}25 \times 10^{-3}$ sec., produced by discharge of a 0·5 μF. condenser through a total external resistance of 2500 ohms. The second stimulus can be applied either at the same point as the first or at a nearby point. There is an absolute refractory period of 70–75 msec. and the relative refractory period is discernible up to about 120 msec. after the first stimulus. Results are shown in Fig. 4, together with the results found for *Calliactis* by Pantin (1935).

The existence of the refractory period implies that an all-or-nothing impulse is initiated under the first electrode, and the observation that the second stimulus can be at a point distant from the first shows that the impulse has this property along the conducting pathway.

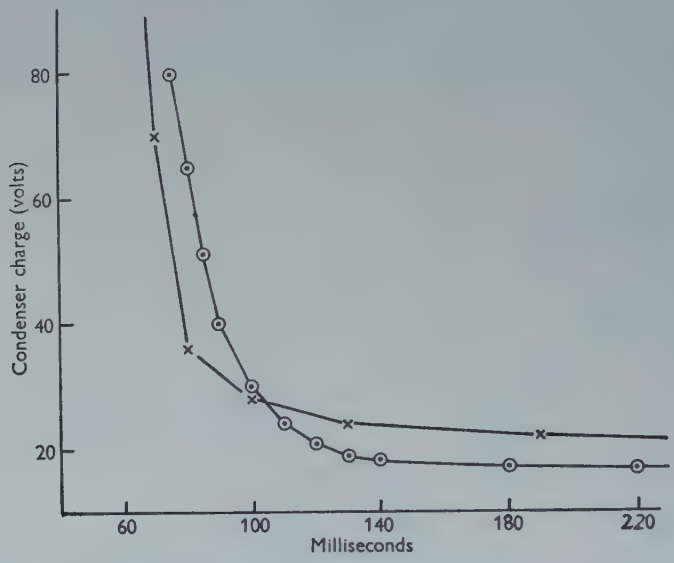

Fig. 4. The relation between intensity of stimulation and refractory period of the through-conducting system. ⊙, *Cerianthus*, the stimuli being applied to a tentacle; ×, *Calliactis* from Pantin (1935).

VELOCITY OF TRANSMISSION

The velocity of transmission of the through-conducting system was measured along the column by taking into account the apparent increase in the refractory period as the second shock is applied progressively further away from the first. With a charge of 40 V. the results appeared to be unaffected by local variations in sensitivity. With both electrodes at the same place the refractory periods for six trials were 85, 92, 86, 85, 90, 90 msec. With the same electrodes separated by 6 cm. of the column the apparent refractory periods were 125, 134, 120, 145, 140, 135 msec. This gives an average velocity of transmission of about 1·3 m./sec.

CONTRACTION OF THE ECTODERMAL MUSCLE OF THE COLUMN

Column contraction was recorded with an isotonic lever arranged to pull the muscle with a load of approximately 10 g. The preparation was held by pins or threads and submerged in aerated sea water. Under these conditions the whole animal or a longitudinal strip of body wall would relax and steadily lengthen for the first half hour or so and would then usually show slow irregular changes in length, each of which might take an hour to complete. Some of this slow, apparently spontaneous,

movement may have originated at the mechanical supports, but I consider that the time courses and heights of contractions recorded in this paper represent real physiological changes of the preparation. Some slow movements, particularly at the aboral end, were observable in untouched whole animals kept in glass tubes.

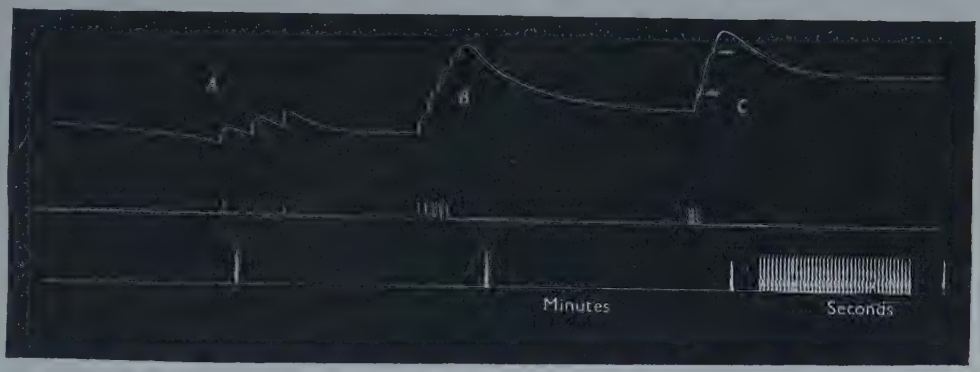

Fig. 5. A kymograph record of longitudinal contractions of the column of an intact *Cerianthus*. This record illustrates the contraction at each shock, the slow relaxation, the staircase of successively smaller steps at constant time interval, and the larger contraction (facilitated) at *C* following stimuli that are very close together. In *C* the steps are indicated.

The twitch of the muscle in response to a single shock is symmetrical on all sides of the column. The relaxation is slow, as shown in Fig. 5. The contraction height of a single twitch is independent of the stimulus strength above threshold. The height of the contraction, as measured by the maximum excursion of the trace on a smoked drum, depends upon the initial length at the moment of stimulation as shown by the points × in Fig. 6. This relation, which is common to many slowly relaxing muscles, appears to have no particular significance here, but must be allowed for when considering the question of temporal summation of successive contractions. As shown in Fig. 5 the second contraction begins at various stages in the course of relaxation after the first contraction; it is reasonable to assume that the height of the second contraction depends upon the initial length in the same way as the first. In Fig. 6 the circles show the extents of second contractions which follow the first after an interval of 1 sec. The agreement between the two sets of points shows that the second contraction is similar to the first, although on the trace it would rise above the first and the extent of the second contraction would be less than the first. A typical trace which illustrates these features is shown in Fig. 5 at *A* and *B*. However, the second contraction is larger than normal for intervals less than 1 sec. as in Fig. 5, *C*. When a second shock is applied at various time intervals after the first, at the same point on the column or tentacle, the resulting total contractions are greatest for intervals of about 100 msec. The curve of the facilitation of contraction, Fig. 7, is comparable with that which Pantin (1935) obtained for *Calliactis*, though more sharply inflected. The present observations on *Cerianthus* do not provide any information as to the site of the mechanism of facilitation because

they do not show whether it occurs in the conducting system or in the contraction process; in *Calliactis* this distinction was possible.

Contractions which are much slower, asymmetrical, and often restricted to one end of the column are part of the normal behaviour of *Cerianthus*. Specimens kept in glass tubes continually make slow bending movements of the end of the column. The asymmetrical bending responses to light and to gravity described by Moore (1927*a*, *b*) are changes of posture which are maintained by the longitudinal ectodermal muscle of the column. As mentioned above, preparations arranged for kymograph recording show slow movements, but it has not been possible to find the cause of these or to discover their relation to the normal behaviour of the whole animal.

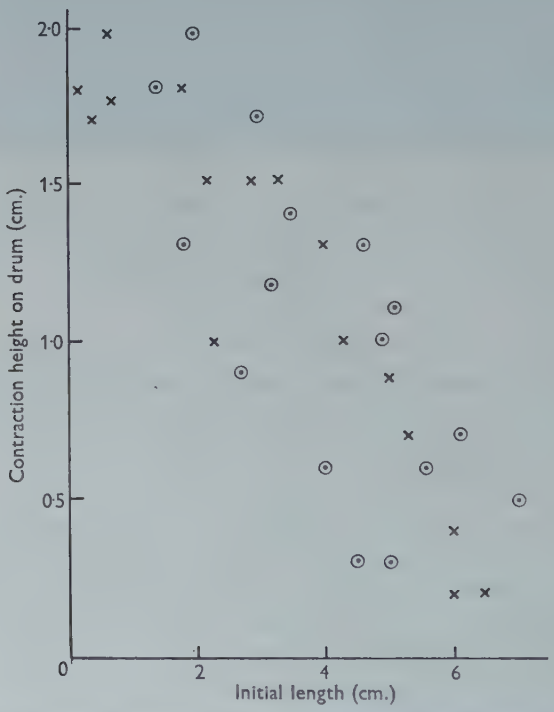

Fig. 6. The extent of the second contraction plotted against the initial length of the muscle: ×, after a delay of at least 20 sec.; ⊙, after 1 sec. delay since the previous stimulus. The ordinates refer to arbitrary measures of the excursion on a kymograph drum.

Longitudinal strips and mounted preparations of the whole column show an asymmetrical slow component of contractions following several electrical stimuli. The responses to the first few shocks are followed by a relaxation that is complete in 1–2 min. As shown in Fig. 8, later contractions may be followed by an abnormally slow relaxation. The slow relaxation occurs all along the length of the preparation. In a preparation of two longitudinal strips connected by a transverse bridge in the form of a U the rapid contractions and relaxations are similar in the two strips. The slower relaxation is not propagated from the strip in which it originates. A typical

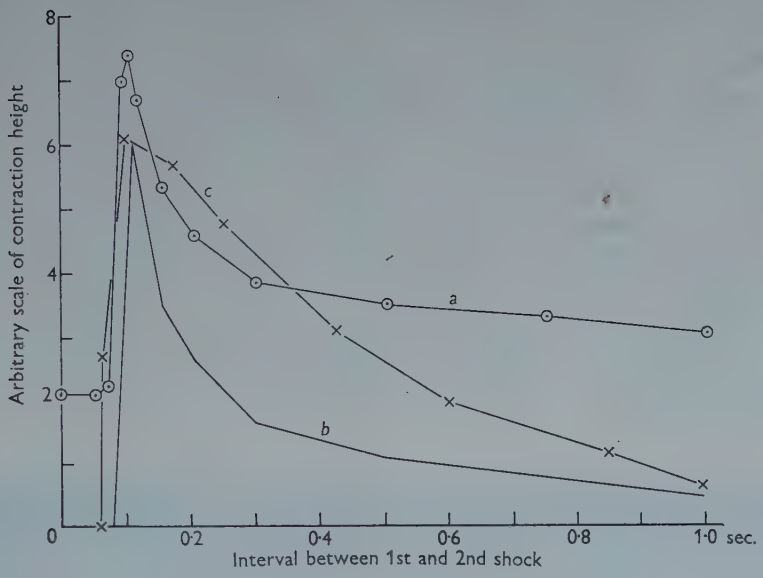

Fig. 7. Facilitation of contraction in *Cerianthus*. (*a*) The relation between the interval between two shocks and the height of the resulting two contractions taken together; (*b*) the height of the component due to facilitation alone. This curve is derived from (*a*) by deduction of the heights expected if each shock had occurred separately; (*c*) Pantin's (1935) results on *Calliactis* redrawn so that the height of the peak and the base line are similar to those of curve (*b*) on the arbitrary scale of contraction height. The anemone contracts only at the second shock and (*c*) is comparable with (*b*). Note that the facilitation is at a maximum at similar positions on the time scale in (*b*) and (*c*), but falls off relatively more rapidly in *Cerianthus*.

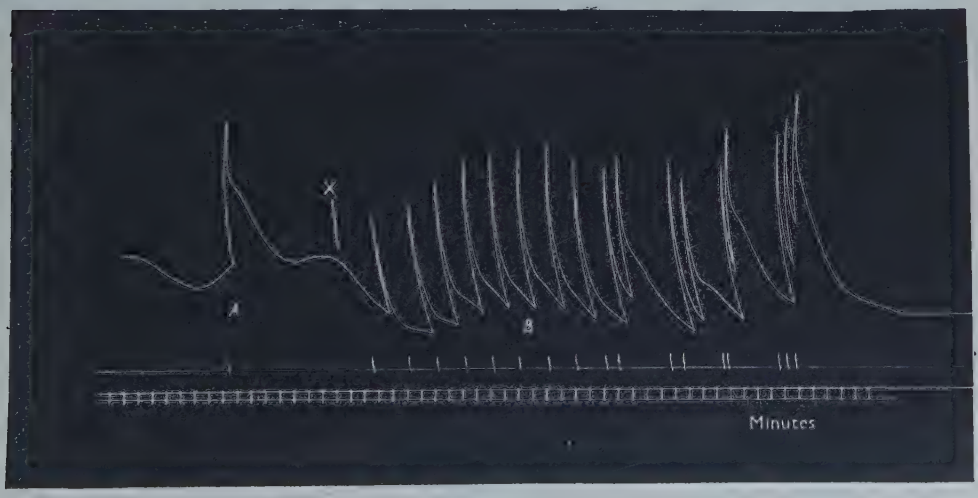

Fig. 8. Various types of contraction in *Cerianthus*. *A*, Response to a single electrical stimulus during a slow contraction which was elicited by mechanical stimulation; *B*, typical quick responses. Note the delayed relaxation in *A* and in the later quick contractions.

trace is shown in Fig. 9. Ten shocks at 2 per sec. is an adequate stimulus to produce a contraction which relaxes slowly.

The muscle is clearly capable of two distinguishable responses; the twitch followed by the delayed relaxation is a combination of these two. Both responses are propagated over the muscle, for the whole length of it responds to a stimulus at one point. These two responses are co-ordinated by distinct mechanisms since either fast or slow contractions occur separately. The picture which emerges of the co-ordination of the slow contractions following stimulation does not agree with all observations of the slow movements of whole animals. The responses to light and to gravity appear to be direct local responses, not propagated along or round the column. The characteristic movement of the aboral ends of animals in glass tubes is a slow contraction which is co-ordinated round the animal, and forms a terminal bulb: this bulb has an obvious functional significance as a movable holdfast in the animal's natural tube.

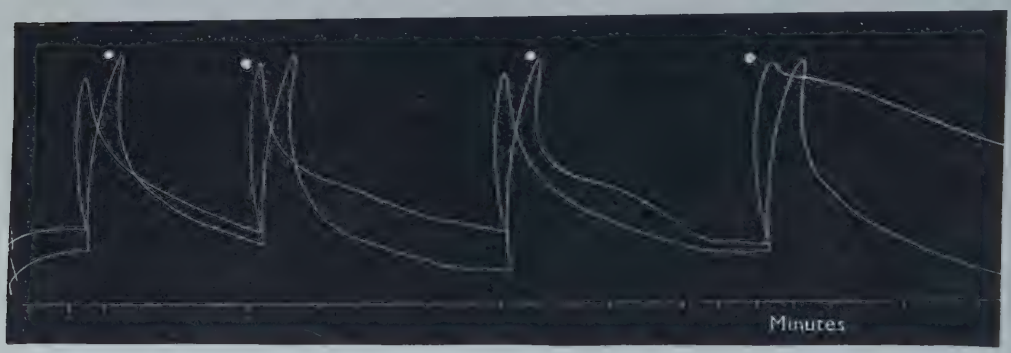

Fig. 9. Contractions of two strips of longitudinal muscle which were joined by a transverse bridge at the aboral end. The stimulus, 20 shocks at 2/sec., was applied to the oral end of one strip or the other as indicated by the white dots. In addition to the symmetrical quick contraction, the stimulated strip alone shows a delayed relaxation which becomes more pronounced as the experiment proceeds.

DISCUSSION

Previous histological work

The ectoderm of the column of *Cerianthus* consists mainly of glandular cells, nematocysts, and the cell bodies of the underlying longitudinal muscle fibres. The contractile fibres lie in radially arranged longitudinal lamellae which are folds of the underlying mesogloea. Below the superficial layer of cell bodies lies a plexus of thin fibres which run parallel to the surface. Some authors, for example, Hyman (1940), have taken these to be nerve fibres but present knowledge of their connexions and histological structure is far from satisfactory. Dr Torelli, the present authority, doubts (1957) the nervous nature of this plexus and supposes it to be connective tissue. She describes (1952) nerve fibres which run over the lamellae of the muscle fibres in the position expected of a motor innervation, but is unable to find the cell bodies of these fibres, or any other ganglion cells. These fibres over the muscle

lamellae (and in the above subepidermal plexus) are very small compared with the nerve nets which are the through-conducting systems of *Metridium* (Pantin, 1952) and *Aurelia* (Schäfer, 1878). After much histological work on *Cerianthus* by a number of authors with varied techniques no fibres or cells which can reasonably be identified with the through-conducting system have been described.

CONCLUSIONS

If the histological evidence is accepted the only fair conclusion from the physiological observations is that the through-conducting system is the ectodermal muscle itself. If so, this would be an exception among coelenterates so far studied and the simplest example of muscular co-ordination yet discovered among multicellular animals. The velocity of conduction, the refractory period and the chronaxie would then be properties of the muscle, despite the quantitative agreement of the first two of these in *Cerianthus* and in the mesenteric system of *Calliactis*, which is accepted as being co-ordinated by a nerve net (Pantin, 1952). But I think that the above conclusion, based upon indirect physiological and negative histological evidence, is premature and would be acceptable only after a direct observation of propagation from muscle fibre to muscle fibre.

The interpretation of results obtained by standard physiological methods of gross stimulation is necessarily severely limited since even if a nerve net is found by histological methods, further evidence is required before it can be accepted as one of the pathways of conduction. Limitations of technique have restricted most studies of nerve nets so that physiological and histological information is obtained independently. In general there is so far no direct evidence that the histologically observed fibres are concerned in the behaviour or that physiological properties can be ascribed to a particular axon structure. There are two exceptions, the physiologically distinct specialized giant fibre systems of actinians (Pantin, 1952) and scyphozoans (Horridge, 1954). In the former the identification is based on the agreement between the fibre orientation in the mesenteric nerve net and the anisotropic distribution of the velocity of conduction in this region; in the latter it was possible to obtain physiological information about a cell which at the same time was identified microscopically.

In the present study I have endeavoured to find the limits of the interpretation which can be placed on the classical experimental methods of gross electrical stimulation. In *Cerianthus*, since a net of definite nerve axons has not yet been found, the position is not complicated by the assumption that the co-ordinating pathway is located in a nerve net. The later discovery of the existence of a net would not alone justify the assumption that it is the site of one of the physiological pathways. Circumstantial evidence, such as directionality, specific connexions to the muscles, or a limited regional distribution would allow a tentative correlation; direct physiological micro-methods could be conclusive.

SUMMARY

1. The responses of *Cerianthus membranaceus* to electrical and mechanical stimulation are described.

2. The longitudinal muscle of the column ectoderm responds with a symmetrical contraction to a single shock from a condenser discharge applied anywhere on the animal. The response is independent of the strength of the stimulus above threshold and is co-ordinated by a through-conducting system which propagates an impulse at about 1·3 m./sec. followed by an absolute refractory period of 70 msec. at 21–23° C.

3. The strength-duration curve of the excitatory process, the relative refractory period curve and the curve of facilitation of contraction are given and compared with corresponding results from actinians.

4. Slow responses of the tentacles and column muscle are described.

5. The physiological observations are discussed in relation to the histological background. The conclusion that the physiological pathway cannot yet be histologically identified is discussed as part of the general problem of the analysis of the coelenterate nerve net.

I should like to thank Dr P. Dohrn and his staff at Naples for their kind interest and for the use of the facilities of the Stazione Zoologica.

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THE RELATION OF OXYGEN CONSUMPTION TO BODY SIZE AND TO TEMPERATURE IN THE LARVAE OF *CHIRONOMUS RIPARIUS* MEIGEN

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INTRODUCTION

The experiments described in the present paper were undertaken so that an assessment could be made of the importance of these larvae in the oxygen balance of polluted streams (Phelps, 1944), where under certain conditions they are very abundant (Edwards, 1957). The effect of the oxygen concentration of the water upon the oxygen consumption of fully grown *Chironomus* larvae has been studied by Ewer (1942) and Walshe-Maetz (1953). The influence of two other factors of primary importance in determining the rate of oxygen consumption, namely, body size and environmental temperature, have been investigated in the present study; work has been confined to 3rd- and 4th-instar larvae and to only two environmental temperatures.

The investigation may be of interest, however, from a more general viewpoint for it is possible with this species, as it is to some extent with the majority of the aquatic arthropods, to distinguish experimentally between an increase in size, indicated by total weight, volume and surface area measurements, and growth expressed in terms of the weight of body constituents other than water (dry weight). The former expression of growth is generally discontinuous whilst the latter is continuous (Teissier, 1931). In consequence of this type of growth pattern, the relationship of oxygen consumption to surface area can be separated from the relationship of oxygen consumption to an exponential function of dry weight. Generally such a separation may be made, by a statistical treatment of the data, only when the exponential function relating oxygen consumption to weight is sufficiently different from that relating surface area to weight. The variability of biological material and the limited size range of most experimental animals are such that a satisfactorily conclusive separation is rarely possible (see Kleiber, 1947).

MATERIALS AND METHODS

Specimens were obtained from the Maple Lodge Effluent Channel, Rickmansworth, Hertfordshire, and placed in aerated water containing effluent mud.

Oxygen consumption

Larvae were conditioned at the test temperature ± 2° C. for at least 24 hr. before testing, mud being present in the conditioning tanks. Oxygen consumption determinations were made principally in October and November 1956, when the effluent temperature was approximately 15° C. The investigation was confined to 3rd- and 4th-instar larvae, and two test temperatures, 10 and 20° C., were used.

Measurements were made with a respirometer of the Warburg constant-volume type. The respirometer flasks described an arc of 1·43 cm. at a rate of 86 complete swings per minute. After a steady state had been reached, the rate of oxygen uptake over 5 hr. was recorded. Groups of from seven to thirty-three larvae were placed in each flask, the number depending upon the body size of each group, and the groups being selected by eye for size uniformity. The respirometer was fitted with a hood which excluded most of the light.

Larvae were subsequently placed on filter-paper to remove surface moisture and weighed (wet weight). They were then dried overnight at 105° C. and reweighed (dry weight). The average weight for each group was determined.

Specific gravity

The specific gravity of larvae was measured by immersing them in solutions of sodium chloride and of sucrose. In Fig. 7 which shows the relationship between specific gravity and wet weight, the close agreement between sodium chloride and sucrose determinations suggests that salt absorption does not change the specific gravity of the larvae appreciably during the period under test. Larvae were divided into groups of similar size, the average body weight of each group being determined. The specific gravity of each size group was measured by immersing several larvae in each of a series of solutions ranging in specific gravity from 1·01 to 1·06. The number of larvae at the surface, suspended in the solution, or resting on the bottom of the vessel, was recorded 1 min. after immersion. This method, though perhaps not as accurate as the displacement method of density determination described by Lowndes (1942) for marine animals, is simpler and quicker.

Linear dimensions

The lengths of larvae were measured by means of a micrometer eyepiece, the larvae being first immersed in 0·5% chloral hydrate to reduce body movements. Larvae were prevented from bending by placing them on a cavity slide, the width of the cavity being approximately that of the larvae. The average length and average wet weight of groups of ten larvae, selected by eye for size uniformity, were recorded. The measured length included the head but not the posterior pseudopods.

RESULTS

Oxygen consumption

An equation of the form $R=aW^b$ (where R is the oxygen consumption of an organism of weight W and a and b are constants) is often given as an expression of the relationship between oxygen consumption and body weight. A plot of the logarithmic transformation, $\log R=\log a+b \log W$, gives a straight line, the slope of which denotes b, relating oxygen consumption to weight. The equation is sometimes expressed in the form $r=aW^{(b-1)}$, where $r=R/W$, the oxygen consumption per unit weight.

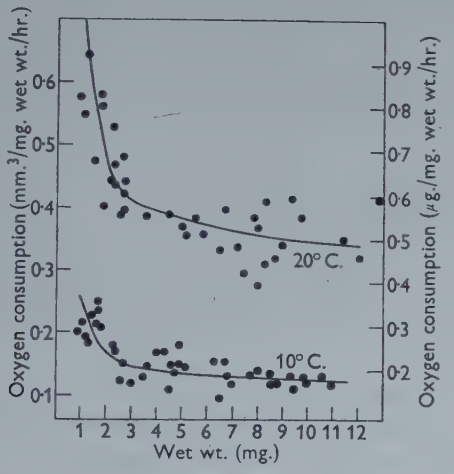

Fig. 1

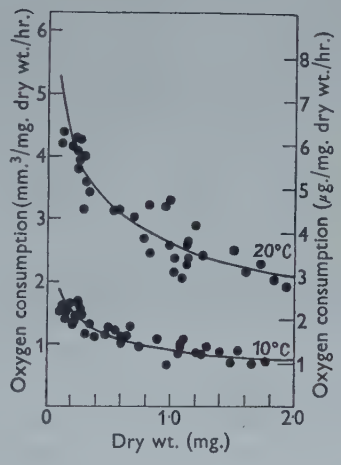

Fig. 2

Fig. 1. Oxygen consumption per unit wet weight plotted against wet weight.

Fig. 2. Oxygen consumption per unit dry weight plotted against dry weight.

Fig. 1 shows the values for oxygen consumption per unit wet weight plotted against wet weight at 10 and 20° C. The wet weight varies between 0·89 and 12·82 mg., and over this size range the oxygen consumption per unit weight of the smallest larvae is about twice that of the largest. Fig. 2 shows the values for oxygen consumption per unit dry weight plotted against dry weight. It is clear from an inspection of the data that there is a progressive decrease in oxygen consumption per unit weight as the size of the animal increases.

Logarithmic transformations of the data shown in Fig. 1 are drawn in Fig. 3. These are clearly not straight lines, that is, b has no constant value and the relationship $R=aW^b$ does not hold when wet weight is used as a measure of body size. On the other hand, logarithmic transformations based on the dry-weight data, shown in Fig. 4, suggest that b has a fixed value. Regression analyses gave coefficients of $-0{\cdot}29$ ($\pm 0{\cdot}023$) at 10° C. and $-0{\cdot}30$ ($\pm 0{\cdot}025$) at 20° C. The oxygen

consumption per unit weight is proportional to these powers of the dry weight, and the total oxygen consumption is proportional to the 0·71 power of the dry weight at 10° C. and to the 0·70 power at 20° C.

The larvae consume oxygen 2·6 times as fast at 20° C. as at 10° C. over the size-range studied. The results suggest that the Q_{10} value is not dependent upon size, the difference in the regression coefficients being insignificant and the results of a 't' test giving $P=0{\cdot}3$.

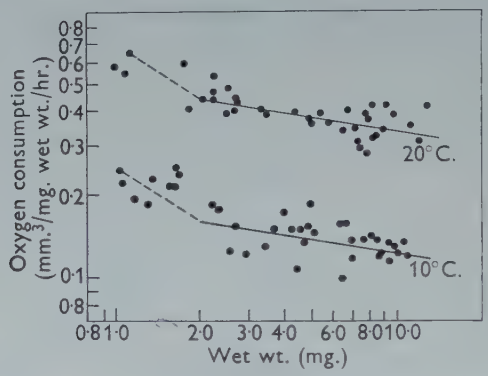

Fig. 3. Log oxygen consumption per unit wet weight plotted against log wet weight. Continuous lines, representing the oxygen consumption of 4th-instar larvae, are drawn from calculations based on regression lines in Figs. 4 and 6.

The oxygen consumptions of several groups of larvae were determined at twice the normal amplitude of shaking of the respirometer flasks, to ascertain the effect of shaking upon the rate of oxygen consumption (Table 1). A regression analysis using the method of concomitant observations (Mather, 1943) applied to the data suggests that the difference in amplitude of shaking did not influence the oxygen consumption of the larvae, the results of a 't' test giving $P=0{\cdot}6$.

The experiments concerning the effect of shaking on the rate of oxygen consumption were carried out in August 1957, whereas the remainder of the oxygen-consumption determinations were made in October and November 1956. The results of an analysis of covariance suggest that the oxygen consumption per unit dry weight of the 'summer' larvae is significantly higher than that of the 'autumn' larvae ($P<0{\cdot}001$), whereas the slopes of the regression lines for 'summer' and

Table 1. *Effect of amplitude of shaking on oxygen consumption*

	Oxygen consumption at 20° C. (mm.³/g. wet wt./hr.)								
Normal shaking amplitude	487	457	436	332	390	380	410	419	422
Normal shaking amplitude × 2	528	466	391	390	481	399	366	409	418
Average wet wt. (mg.)	6·23	7·96	10·13	7·23	7·16	11·71	9·23	10·24	9·41

Average wet weights were converted to dry weights for comparing seasonal oxygen consumption rates (Fig. 4) using the relationship between wet and dry weights shown in Fig. 6.

'autumn' larvae (see Fig. 4) are not different ($P=0{\cdot}3$). The data for both amplitudes of shaking were grouped in these comparisons of seasonal rates of oxygen consumption.

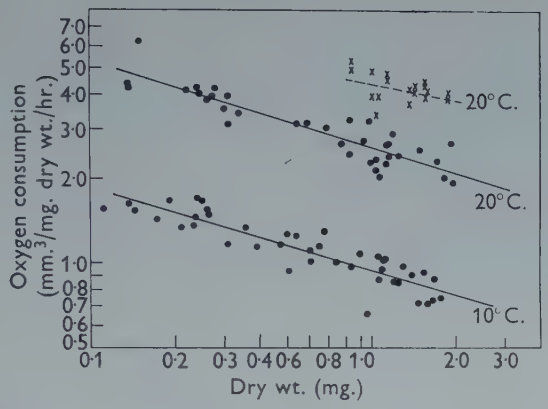

Fig. 4. Log oxygen consumption per unit dry weight plotted against log dry weight. ×, August determinations; ●, October–November determinations.

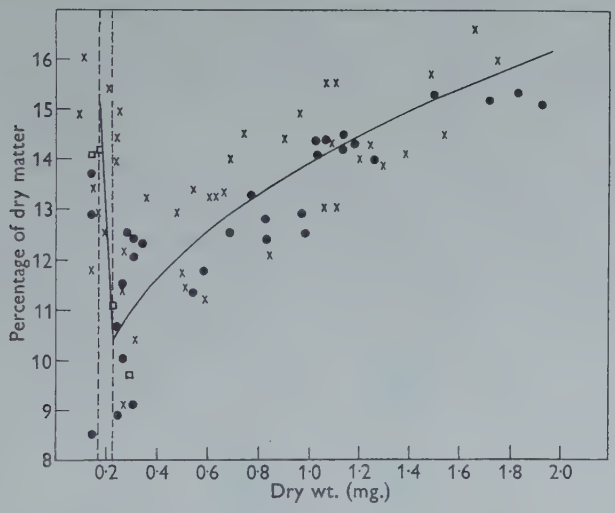

Fig. 5. Change in percentage of dry matter with growth. ×, larvae from oxygen consumption determinations at 10° C.; ●, larvae from oxygen consumption determinations at 20° C.; □, data from Table 2.

Percentage of dry matter

The change in the percentage of dry matter with size, indicated by the disparity between the oxygen-consumption relationships based on wet and dry weights, is shown in Fig. 5. The percentage of dry matter decreases suddenly when larvae reach a weight of approximately 0·2 mg. dry weight. Subsequent growth is associated with a gradual increase in the percentage of dry matter. Geng (1925) found that larvae of *Chironomus thummi* K (=*riparius* Meigen) weighing 0·67 mg. dry

weight contained 12·82% dry matter, whilst Karsinkin (1935) found a value of 17·94% for larvae weighing 1·35 mg. dry weight. Geng's value lies close to the curve drawn in Fig. 5.

Larvae weighing between 0·1 and 0·3 mg. dry weight were separated into their respective instars using head size (Tanaka, 1939), grouped, and the percentage of dry matter was determined. Table 2, summarizing these data, indicates that moulting occurs when larvae weigh between 0·17 and 0·23 mg. dry weight. The initial increase in body weight following the moult is principally the result of water intake, this intake being reflected by the change in percentage of dry matter.

The relation between dry weight and wet weight throughout growth in the 4th instar is plotted logarithmically in Fig. 6. The dry weight varies as the 1·186 power (±0·022) of the wet weight.

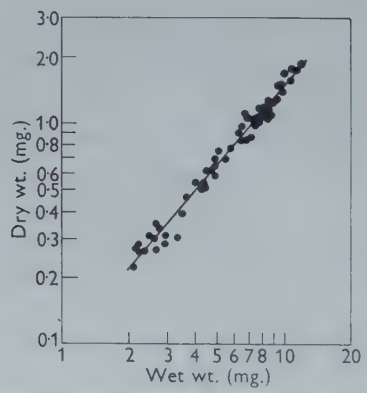

Fig. 6. Log dry weight plotted against log wet weight for 4th-instar larvae.

Table 2. *Change in percentage of dry matter associated with the third larval moult*

Instar	Average wet wt. (mg.)	Average dry wt. (mg.)	Percentage dry matter
3	1·22	0·1735	14·2
3	1·02	0·1433	14·1
4	2·096	0·223	11·1
4	2·955	0·286	9·7

Although the relationship $R = aW^b$ does not hold for larvae of *C. riparius* over the whole of the size range studied when body size is expressed as wet weight (see Fig. 3), a constant value of b may be found for the 4th instar since it has been shown that (*a*) the oxygen consumption varies as the 0·7 power of the dry weight, and (*b*) the dry weight varies as the 1·186 power of the wet weight.

Thus, within the 4th instar oxygen consumption varies as the 0·83 power (1·186 × 0·70) of the wet weight. Though the value of b appears to be constant within an instar, moulting brings about a change in the value of the constant a, so

that, when log oxygen consumption is plotted against log wet weight over a size range associated with two or more instars, the relationship cannot be represented by a single straight line (Fig. 3).

Specific gravity

An increase of specific gravity from about 1·026 to 1·043 occurs during growth from 3 to 11 mg. wet weight (see Fig. 7). This increase reflects the increase in percentage of dry matter which takes place during growth within the 4th larval instar.

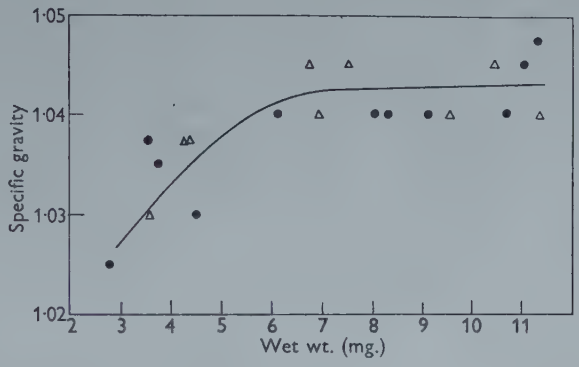

Fig. 7. Change in specific gravity of 4th-instar larvae with growth. △, sucrose determinations; ●, sodium chloride determinations.

Table 3. *Volume, wet weight, specific gravity and dry weight of early 4th-instar larvae as compared with late 4th-instar larvae*

	Dry wt. (mg.)	Wet wt. (mg.)	S.G.	Volume ($mm.^3$)
Early	0·3	2·74	1·026	2·67
Late	1·8	11·32	1·043	10·86
Ratio $\frac{\text{late}}{\text{early}}$	6	4·13	—	4·06

$$\frac{\text{Ratio (wet wt.)} - \text{ratio (volume)}}{\text{Ratio (wet wt.)}} \times 100 = 1{\cdot}7.$$

$$\frac{\text{Ratio (dry wt.)} - \text{ratio (wet wt.)}}{\text{Ratio (wet wt.)}} \times 100 = 45{\cdot}3.$$

Table 3 shows how closely volume is proportional to wet weight, the discrepancy amounting to no more than 1·7 %, whereas dry weight is not proportional to wet weight, the discrepancy being 45·3 %.

Surface area estimates

If one assumes that the shape of a *C. riparius* larva approximates to a cylinder, the surface area of such larvae may be calculated from length and volume measurements, the latter being calculated from weight and specific gravity determinations.

One of the more important errors in this approximation of surface area is probably that resulting from the omission of the ventral gills, whose surface area is larger compared with their weight. For the present study, however, where the surface area is being considered primarily in its relationship to oxygen consumption, the surface area of the ventral gills is best omitted in view of Fox's observation that they do not absorb oxygen as does the rest of the body surface (Fox, 1921; Thorpe, 1933). The area through which oxygen enters, being less than the total surface area, is probably determined more accurately by the cylinder approximation than by actual measurement.

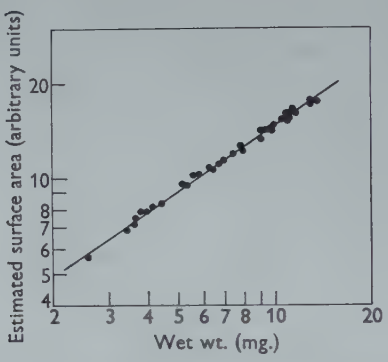

Fig. 8. Log estimated surface area plotted against log wet weight for 4th-instar larvae.

Fig. 8 shows the logarithmic transformation of estimated surface area plotted against wet weight for 4th-instar larvae. The surface area is proportional to the 0·70 power of wet weight. The standard error has not been calculated because the variables are not independent, the surface area estimates being calculated partly from weight.

In Fig. 9 where oxygen consumption at 20° C. is plotted against estimated surface area it is evident that the relationship is not one of simple proportionality. The oxygen consumption per unit area increases with increase in size (throughout the 4th instar). Values for the total oxygen consumption of 3rd-instar larvae have not been included in Fig. 8 as the specific gravity and the relationship between length and weight have only been investigated for the 4th-larval instar.

DISCUSSION

The relationship between size and respiration rate has been extensively reviewed in recent years (Weymouth *et al.* 1944; Kleiber, 1947; Ellenby, 1951; Zeuthen, 1953, 1955; Bertalanffy, 1957). It has been widely observed that the respiration rate falls with increasing size and these observations have some justification on thermodynamic grounds (Prigogine & Wiame, 1946). Generally, *b*, the exponent relating respiration and size, has a fixed value for wide size ranges of many organisms, though Zeuthen (1955) suggests that it is best considered as a tangent to

a more complicated curve. Kleiber (1947), discussing the theoretical validity of the Surface Law (Rubner, 1883), concludes that there is no satisfactory basis for the acceptance of a causal relationship between surface area and respiration, especially with poikilothermic animals. Zeuthen (1953) 'considers that the surface concept has dominated our thinking in this field far too much', though as Needham (1942)

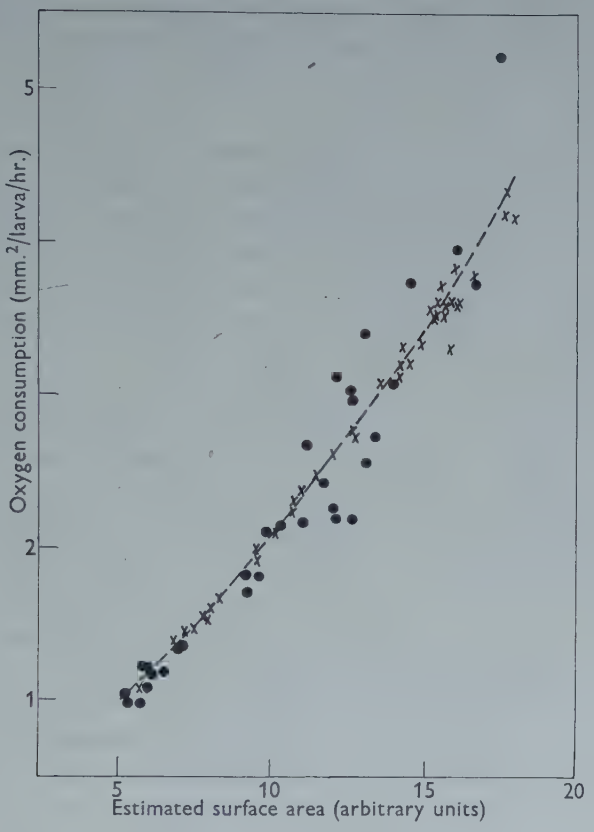

Fig. 9. Oxygen consumption plotted against estimated surface area for 4th-instar larvae. ●, values calculated from measurements of oxygen consumption at 20° C. recorded in Fig. 3, the surface area estimates being assessed from the relationship between weight and surface area shown in Fig. 8; ×, values calculated from surface area estimates recorded in Fig. 8, the oxygen consumption being calculated from the relationship between weight and oxygen consumption at 20° C. shown in Fig. 3.

points out, the 'surface factor' must not be excluded. The 'surface factor' may be of greater relevance if it is not restricted to the external body surface. Rensch (1948) describes how certain changes in tissue structure and cell shape are associated with changes in body size, and such histomorphological changes may be of importance in our more complete understanding of the relationship between body size and rate of respiration.

Ellenby (1945, 1951) has pointed out that there is no *a priori* reason why the surface areas of a series of bodies of differing size should be proportional to the two-thirds power of their weights. There is no justification for the assumption that the Surface Law applies when it has been shown that b has a value not significantly different from two-thirds (Gilchrist, 1956), unless it has been demonstrated that there are no changes in shape and specific gravity. It is also true, however, that the rejection of the Surface Law on the grounds that b differs from two-thirds is equally faulty. This association between surface area and the two-thirds power of the weight has led to much of the disorder now prevailing in this field.

If one were looking for a poikilothermic animal to demonstrate the relationship between surface area and oxygen consumption one might well select the larva of *C. riparius* which has an extremely rudimentary tracheal system (Miall & Hammond, 1900) and which absorbs oxygen through the general body surface (Fox, 1921). The results of the present investigation suggest, however, that the oxygen consumption is not proportional to the estimated surface area, but varies as the 0·7 power of the dry weight of the body.

It seems best to consider the relationship between size and respiration as a further demonstration of the heterauxesis theory (Needham, 1942). This states that when the magnitude of a part of an organism, either chemical or anatomical (y) is compared with its totality (x), a relationship of the form $y=cx^d$, where c and d are constants, is frequently observed. Needham suggests that the fall in metabolic rate is best regarded as a change in the relative proportion of respiring protoplasm and inert material in the cells. Several workers have found falling metabolic rates in tissues taken from animals of increasing size (Weymouth *et al.* 1944; Krebs, 1950), and Rosenthal & Drabkin (1943) observed that cytochrome c concentrations of mammalian epithelium decreased with increasing body weight.

Bertalanffy (1951) has carried the analysis of the size/respiration relationship somewhat further and has divided animals into three metabolic groups according to the values of b. Bertalanffy (1951) and Bertalanffy & Krywienczyk (1953) have suggested that some taxonomic groups are homogeneous in respect of the value of b. It is also suggested that the metabolic groups have characteristic forms of growth. Some evidence does not accord with Bertalanffy's views (see Zeuthen, 1955). Mann (1956) records values of b varying from 0·695 to 1·06 for five species of freshwater leeches; two closely related species *Erpobdella octoculata* and *E. testacea* having values of 1·06 and 0·81. It has been shown, however, that three of the species studied have a similar type of growth (Mann, 1953, 1957). According to Bertalanffy (1951) the metabolic rate of insect larvae is proportional to body weight (see also Edwards in Roeder, 1953); this is at variance with the conclusions of the present investigation.

Ewer (1942) measured the oxygen consumption of fully grown *Chironomus* larvae of the *plumosus* group (subsequently identified as *C. cingulatus* Mg and *C. riparius*

Mg) at 17° C., using the syringe and micro-Winkler method described by Fox & Wingfield (1938). Although the weights of the larvae and the season of the year are not given, a comparison of the results is of some interest. For fully grown winter larvae (10 mg. wet weight), assuming a linear relationship between temperature and oxygen consumption between 10 and 20° C., the calculated oxygen consumption at 17° C. would be about 280 $mm.^3$/g. wet wt./hr. Ewer found an oxygen consumption of about 190 $mm.^3$/g. wet wt./hr. The discrepancy may be attributed to differences of method or material, for the findings of Tanaka (1939) on the temperature/growth relationship of *C. dorsalis* Mg, suggest that the temperature relation is approximately linear over this temperature range ($Q_{10}=1{\cdot}53$). Walshe-Maetz (1953) found discernible differences between the oxygen consumption rates of two subspecies of *C. plumosus* L. It is unlikely, however, that differences between closely allied species could satisfactorily account for a discrepancy of such magnitude. It seems possible that the difference in methods employed in the oxygen consumption determination may be largely responsible for the apparent difference between the oxygen consumption rates. Both methods are extensively used in respiration studies of aquatic animals, and comparative studies similar to that made by Wilder (1937) with salamanders, would seem to be worthwhile.

Estimates of the total oxygen consumption of natural populations based on laboratory determinations must be treated with caution. Walshe-Maetz (1953) has clearly shown how the degree of artificiality in test conditions influences the oxygen consumption rate. Estimates made from oxygen consumption values presented in this paper agree well, however, with observed changes in the oxygen uptake of stream muds when known numbers and weights of larvae are added to them (Edwards, unpublished).

Seasonal adjustments in oxygen consumption rates similar to those described by Edwards & Irving (1943) for *Emerita talpoida*, where rates in winter are higher than those in summer at comparable temperatures, have not been found with *Chironomus riparius*. It seems that the oxygen consumption rate at 20° C. is higher in summer than in the winter. The depression of metabolism in the winter may be regarded as evidence of an arrested development of the overwintering generation (Lees, 1955) which remains at the larval stage from October until March. There are about seven generations between March and October.*

With *C. riparius* larvae, the relation between oxygen consumption and size is not influenced by temperature. Whitney (1942) recorded a similar independence for *Crenobia* (*Planaria*) *alpina*. Hotovy (1938), working with *Triops cancriformis*, found that the size factor diminished with decreasing temperatures. The usual relationship, $R=aW^b$, does not fit Hotovy's data, however, especially at the lower temperatures. Edwards (1946), discussing the influence of temperature upon the oxygen consumption of *Talorchestia* and *Melanotus*, concluded that small animals show a greater response to temperature changes than large ones. This conclusion

* See *Freshwater Biological Association Annual Report*, **23**, 1955, p. 38.

was not based on relative values, however, and an inspection of the graphs drawn from the data (Prosser, 1950) suggests that the proportionate response of small animals to a given temperature change was no greater than for large ones.

SUMMARY

1. The oxygen consumption rates of 3rd- and 4th-instar larvae of *Chironomus riparius* have been measured at 10 and 20° C. using a constant-volume respirometer.

2. The oxygen consumption is approximately proportional to the 0·7 power of the dry weight: it is not proportional to the estimated surface area.

3. This relationship between oxygen consumption and dry weight is the same at 10 and at 20° C.

4. The rate of oxygen consumption at 20° C. is greater than at 10° C. by a factor of 2·6.

5. During growth the percentage of dry matter of 4th-instar larvae increases from 10 to 16 and the specific gravity from 1·030 to 1·043.

6. The change in the dry weight/wet weight ratio during the 4th larval instar supports the theory of heterauxesis.

7. At 20° C., 'summer' larvae respire faster than 'winter' larvae.

I wish to thank the Director and Staff of the Water Pollution Research Laboratory, Department of Scientific and Industrial Research, for the help I have received during the course of this work; Miss M. Blakemore and Mr W. P. G. Smith for their valuable assistance, and Miss C. Kipling for the analysis of covariance.

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SURFACE CHARACTERS OF DIVIDING CELLS

I. STATIONARY SURFACE RINGS

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INTRODUCTION

Though many theories have been put forward on the mechanism of cell division, no general agreement has so far been reached. In spite of the fact that each theory brought up various important aspects of the phenomena, such lack of agreement may partly be caused by the inadequacy of data on the stresses acting on the surface of cleaving cells, particularly concerning their magnitudes and the modes of their changes during the process of cell division.

The stress, in the present paper, is considered in terms of displacement of cell parts with reference to the centre of gravity of the cell.

MATERIALS AND METHODS

The eggs of the sea-urchins *Mespilia globulus* and *Hemicentrotus pulcherrimus* were used. In these eggs the mitotic apparatus can clearly be observed through the cytoplasm. The contour of the cell-body is radially symmetrical about the spindle axis, and the cleavage is equal. Accordingly, by choosing eggs in which two astral centres are in the same optical plane, it is easy to find eggs whose spindle axes lie in a strictly horizontal plane.

Since the egg cell shows neither translational nor rotational movement during its first division, it is justifiable to think that the centre of gravity of the egg is at the mid-point between the two astral centres. Once the centre of gravity of the cell is determined, stresses developed during the division process can be expressed as the displacement of cell parts in relation to this centre. This is allowable, because division can be achieved irrespective of any movement of the cell as a whole.

To visualize displacement of the cortex, fine particles of animal charcoal were applied to egg surfaces which had been denuded of the fertilization membrane and hyaline layer according to the technique of Dan, Yanagita & Sugiyama (1937).

The subsequent procedure was as follows: an egg with a horizontal spindle was chosen; the contours of the largest optical section, the positions of particles attached to the contour and those of the astral centres were sketched by a camera lucida at each succeeding stage of division. After completion of the division process such series of sketches were superimposed with reference to the centre of gravity and the spindle axis. In this way, the loci of the particles were obtained.

RESULTS

In Figs. 1 and 2, such superpositions of sketches obtained respectively for *Mespilia globulus* and *Hemicentrotus pulcherrimus* are shown.

On the whole, particles on the furrow surfaces are pulled in, more or less converging to the point of the last attachment of the blastomeres, while particles on the polar surfaces are pushed away in a slightly diverging fashion. But the most surprising thing is that, between the two zones, there are four definite points (in two-dimensional figures) at which all contour sketches intersect. Evidently these points are symmetrical with respect to the spindle axis as well as to the cleavage plane. Therefore, extending this statement to the three-dimensional condition by rotating the sketch around the spindle axis, the four crossing points can be integrated into two rings which are held in common by the egg surfaces at each successive stage of division.

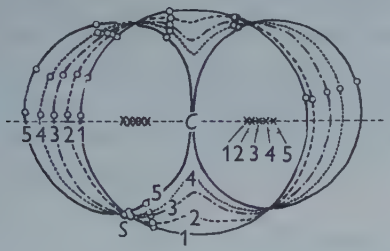

Fig. 1. For explanation see text.

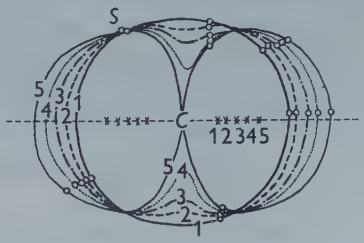

Fig. 2. For explanation see text.

Examination of Figs. 1 and 2 shows that carbon particles which happen to be attached exactly on these rings from the beginning (denoted by 'S') are held stationary at the same position all through division activity. From this fact it is clear that the pair of rings is not only shared by continuously changing cell contours of any stage of cleavage, but is also held absolutely stationary against the spindle co-ordinate with the centre of gravity as the origin of this co-ordinate.

These two rings at the subfurrow region* will hereafter be called 'the stationary surface rings'.

At any rate, as long as the rings remain in the same position, it is beyond any dispute that stresses are balanced at these points (or along these circles, more strictly speaking) at any moment of cleavage, in spite of lack of concrete data on how many components the stresses can be divided into or in what direction and with what magnitude each component is working.

Another fact which should be pointed out in connexion with the figures is the fact that before cleavage the astral centres are situated about 3/8 radius away from the centre of the egg, whereas after the division is complete they are pushed farther away from each other and now lie outside the rings. This means that the astral centres pass through the stationary surface rings during the polar elongation of the cell.

* Furrow, subfurrow, and polar region are defined after Dan *et al.* (1937).

DISCUSSION

As stated above, it has been found that there is a pair of stationary surface rings in the subfurrow region of the dividing sea-urchin egg.

The polar expansion theory of cell division which was proposed by Mitchison (1952) can anticipate the sliding of the whole surface toward the furrow side. But if there are two immobile rings on each side of the furrow region, it may become very difficult to visualize how the movement of the polar region alone can let the furrow surface cut into the cell body.

A reverse situation is encountered by the constricting ring theory of the furrow region advocated by Marsland & Landau (1954). In other words, with the stationary ring interposed, the furrow region and the polar region are more or less clearly separated and it will be very difficult to correlate the behaviour of the two regions at successive stages such as observed by Dan *et al.* (1937).

On the other hand, there seems, at least superficially, to be more possibility of reconciling the present facts with Dan's theory (1943). In the first place, when the convex surface of the furrow region changes towards concavity as it cuts into the cell-body, with its two sides bordered by the stationary rings, the only possible way for this to happen is for the furrow surface first to shrink to flatness and then to reverse its curvature. This corresponds exactly to what Dan calls the initial shrinkage of the furrow region.

In the second place, it is even possible to explain the surface ring by Dan's theory. Dan attributes the main feature of division activity to the pushing away of the two asters by an autonomous elongation of the spindle. He considers that the expansion of the polar surface is due to fountain-like bending of the astral rays as the asters are being pushed toward the polar surface, while the shrinkage of the furrow is due to the pull by the median crossing rays of these same asters as they are departing from the median plane. In other words, simple movement of the asters can give paradoxically opposite effects to the polar and furrow regions, the former being carried away from the median plane while the latter is shrinking toward the median plane. If so, there should be a dividing line between the two which must be in equilibrium between the two opposing forces.

As a corollary, it might be said that the cortical activity theory may have to discover different causes for the polar and the furrow behaviour as the result of the presence of the stationary rings, while the internal activity theory has the advantage of explaining the behaviour of both these regions in terms of a single common cause.

SUMMARY

1. The surface movements during division have been studied by marking the naked surface of the sea-urchin egg with charcoal particles.

2. The contours of the largest optical section, the positions of the particles thereon and the positions of the astral centres are recorded in a series of camera lucida drawings.

3. The drawings are then superimposed, the centre of gravity and spindle axis being used for reference.

4. It is thereby shown that there are two surface rings which remain in the same positions throughout the whole process of division.

5. It is concluded that these rings indicate regions where stresses remain balanced during division.

The writer wishes to thank Professor K. Dan for much valuable advice and discussions. He is greatly indebted to Misaki Marine Biological Station for giving him facilities in the Research Laboratory during this investigation. The writer would also like to express his gratitude to Rikkyo Upper Secondary School for generous financial support.

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ON 'THE STATIONARY SURFACE RING' IN HEART-SHAPED CLEAVAGE

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In the foregoing paper, Ishizaka (1958) succeeded in demonstrating the presence of a pair of ring-zones in the cortex of sea-urchin eggs which remain absolutely stationary with reference to the co-ordinates outside the egg through successive changes of the form of dividing ova. The purpose of the present paper is to see whether or not a similar situation exists in the case of cells cleaving in a heart shape; i.e. in cells in which the cleavage furrow of one side appears earlier than that of the opposite side.

As examples of heart-shaped cleavage, the eggs of the sand dollar, *Astriclypeus manni*, and those of the medusa, *Spirocodon saltatrix*, were used. In these eggs, the cleavage furrow appears first at the animal pole; hence the cell takes a heart shape at some moment of the cleavage. The primary cause of the heart-shaped cleavage is considered by the author to be the eccentric position taken by the cleavage spindle, which lies closer to the animal pole (Dan & Dan, 1947*a*, *b*). When *Astriclypeus* eggs are observed along the egg axis, however, since the contour of the largest optical section corresponds to the equatorial surface of the egg between the two poles, the cleavage pattern becomes seemingly symmetrical with no sign of the heart shape. When the eggs are observed perpendicularly to the egg axis, the difference in the phase of furrow formation at the opposite poles becomes maximal. Observation from intermediate angles gives various degrees of asymmetry. Besides this, there is a fairly wide range of variation in the eccentricity of the cleavage spindle among different batches of *Astriclypeus* eggs, so that by combining the selection of females and angles of observation, the eccentricity can be continuously varied within a considerable range.

In *Spirocodon* eggs, because of an exceeding degree of eccentricity of the spindle, the incipient blastomeres flatten out considerably at the middle of the division process and all come to lie on the flat side, thus showing only the broad side to the observer. In this case the animal and vegetal poles are clearly in view.

The method employed was practically the same as that adopted by Ishizaka; that is, to draw a series of camera lucida sketches in which changing cell contours, changing positions of the astral centres and of the carbon markings on the egg surface were recorded. These cell contours were then superimposed.

Some technical difficulty not encountered in regular cleavage was found in the case of heart-shaped cleavage. In Ishizaka's case, since the ova used cleaved equally

and symmetrically around the spindle axis, superposition of the spindle axes and the mid-points of the two centres of the sketches did not leave any room for ambiguity concerning the manner of superposition. Consequently the existence of four intersections on the contour is unequivocal. But in the case of the heart-shaped cleavage, as the result of the eccentric position of the mitotic figure, the asters are supposed to rotate around their own centres which, in turn, makes the spindle bend toward the vegetal pole (Dan & Dan 1947*a*, *b*). Consequently it is impossible to resort to a purely objective method of superposition such as that adopted by Ishizaka. For the present case, therefore, assuming that stationary regions may exist at least around the animal pole, the drawings of the eggs were superposed accordingly. In this sense, the following is a simple trial even anticipating an error just to see whether or not such a stationary zone can be established for heart-shaped cleavage.

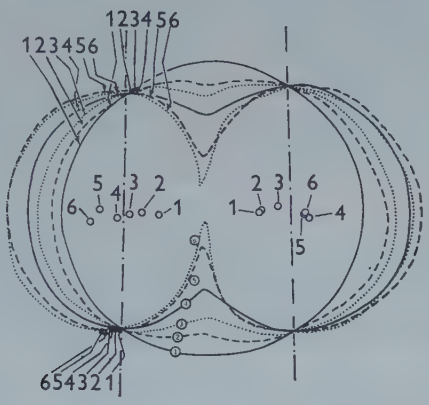

Fig. 1. Superposition of the contour drawings of cleaving egg of *Astriclypeus manni*, a sand dollar, seen along the egg axis. Numbers on the cell periphery are successive positions of adhering kaolin markings and numbers at the centre are those of the astral centres. The contours intersect at four points. Lines connecting two of the points on each incipient blastomere run practically parallel. Loci of the astral centres make roughly two straight lines (eccentricity, 0·5; angle, 4·0°).

The results obtained are illustrated in the figures. As shown in Fig. 1, when the eggs are looked at along the egg axis, that is, when the furrows of the opposite sides appear in a symmetrical fashion, the presence of a pair of stationary surface rings is confirmed beyond any doubt. As was stated before, these skectches were superimposed in such a way as to obtain four stationary intersections. In consequence of such superposition the loci of the astral centres approximate to straight lines. Furthermore, the two planes determined by the stationary rings run almost parallel to each other (in Fig. 1 the two planes deviate from parallel by only 4°).

In the eggs viewed perpendicularly to the egg axis (Fig. 3), if the animal region contours are so superimposed as to give two maximally clear-cut intersection points, the vegetal intersection points vary over a wider range than those encountered in the case of regular cleavage. Yet it is still possible to say that they fall on a relatively narrow portion of the contour. Considering also the case shown in Fig. 2, it is permissible to say that a pair of stationary surface rings occur also for heart-shaped

cleavage with the reservation that they become somewhat blurred in width toward the vegetal side. Tentatively allowing this conclusion, two other features become evident. As the disparity in the phase of furrowing at the animal and vegetal poles increases: (1) the loci of the two astral centres slant upwards and away from each other; (2) the angle included between the two stationary rings becomes more obtuse.

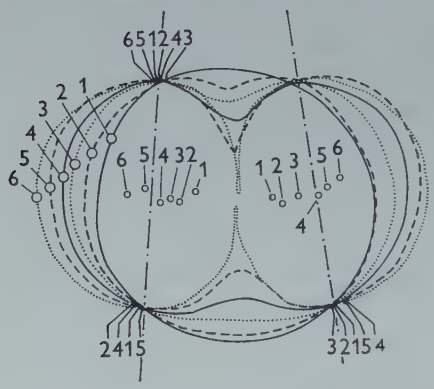

Fig. 2

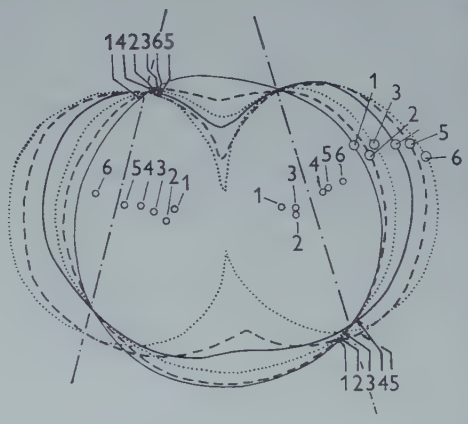

Fig. 3

Fig. 2. Superimposed contour sketches of dividing *Astriclypeus* eggs, seen obliquely to the egg axis. Note asymmetry in the phase of furrowing on the opposite sides. Numbering as in Fig. 1. Lines connecting two intersection points on either side of the furrow converge and the loci of the astral centres tend to slant up at the ends (eccenticity, 0·47; angle, 13·7°).

Fig. 3. Cleaving *Astriclypeus* egg with an extremely eccentric spindle, seen perpendicularly to the egg axis. Note that the inclination of the planes of the stationary surface rings and the slanting of the astral loci are more emphasized than in the previous figures. Note also that the vegetal intersection points shift around during the course of cleavage (eccentricity, 0·43; angle, 31·0°).

Concerning the V-shape of the astral loci, no explanation is available at present. But it may be worth noting that this resembles the bending of the spindle which normally occurs in heart-shaped cleavage.

In order to state in more quantitative terms the proposition contained in the second point, the eccentricity must be expressed more accurately. In the following, the eccentricity is expressed as a ratio, taking the cell diameter as denominator and the distance from the spindle to the nearest cell periphery, the animal pole, as numerator; in other words, when the spindle is central in position, the ratio is 0·5 and the value decreases as the spindle becomes more eccentric. The measurement of eccentricity is not so easy as it may sound because the position of the mitotic apparatus tends to shift toward the centre of the cell as the asters grow larger. Therefore, the effort was made to take the final position of the spindle before the cell departed from the spherical condition.

In connexion with Fig. 1, it was mentioned that the two lines connecting the intersection points run parallel to each other. In this case the apparent position of

the spindle is central (the eccentricity value is 0·5). The egg shown in Fig. 2 has the value of 0·47 and the angle found between the two lines is 13·7°. In the case of Fig. 3, the corresponding values are respectively 0·43 and 23·0–31·0°.

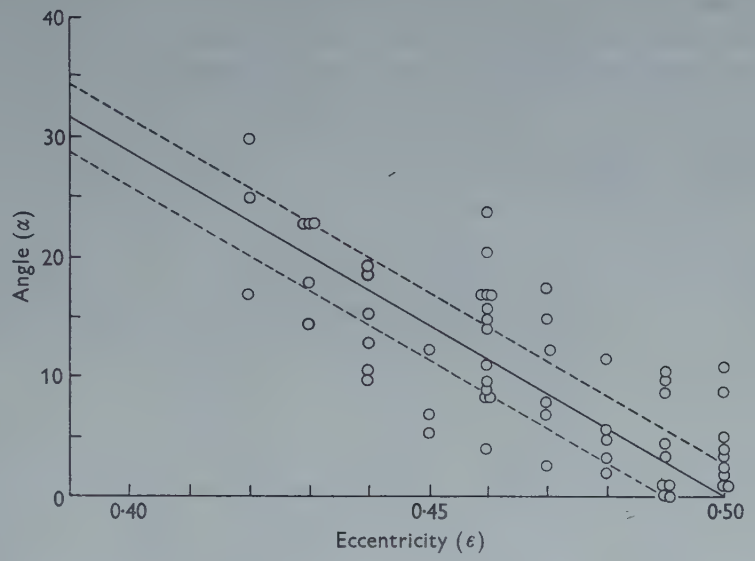

Fig. 4. The data of Table 1 plotted and a straight line fitted to them. The character of the straight line is defined in the text.

Table 1. *Relation between spindle eccentricity and inclination between stationary surface rings*

Eccentricity	Angle in degrees									Average
0·5	4·0	2·5	3·5	11·5	1·0	2·0	5·0	1·0	9·0	4·9
0·49	9·0	10·5	0	10·0	4·5	1·0	3·5	1·0	0	4·9
0·48	3·5	11·5	2·0	5·5	5·0	—	—	—	—	5·5
0·47	12·5	17·5	8·0	2·5	12·5	7·0	15·0	12·5	—	10·9
0·46	20·5	11·0	4·0	17·0	16·0	15·0	17·0	24·0	9·0	—
	14·5	8·5	8·5	9·0	8·5	—	—	—	—	13·0
0·45	5·5	12·5	7·0	—	—	—	—	—	—	8·3
0·44	13·0	10·5	10·0	19·5	19·0	15·5	—	—	—	14·6
0·43	18·0	14·5	23·0	23·0	23·0	—	—	—	—	20·7
0·42	25·0	17·0	30·0	—	—	—	—	—	—	24·0

All the data concerning the angles and the eccentricity are given in Table 1 and plotted in Fig. 4. The plots fall within an inclining belt zone, the two borders of which are almost parallel. The author interprets this as chiefly being due to variation in the eccentricity measurements rather than to error in the measurements of the angles because, in the latter case, the belt would be broader toward the higher values of the angles in a fan shape. On the other hand, it is not surprising to find that the eccentricity varies, as it is not a stable thing itself, and a great deal of chance may be involved in determining the exact stage at which the cell departs from the spherical condition.

If the plots are grouped by 0·01 unit of the eccentricity, the straight line shown in Fig. 4 is obtained, which can be defined as

$$\alpha = \lambda\,(0{\cdot}50 - \epsilon),$$

α is angle between the two stationary rings; ϵ is eccentricity of the spindle. λ was found to be 285 by the least square; the mean square being

$$\overline{\Delta^2} = \{\overline{\alpha - \lambda\,(0{\cdot}50 - \epsilon)}\}^2 = 5{\cdot}3^2,$$

which is shown as broken lines in Fig. 4.

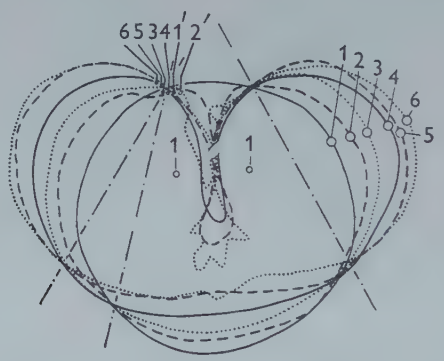

Fig. 5. Superposition of contour sketches of cleaving egg of the medusa, *Spirocodon saltatrix*, seen perpendicularly to the egg axis. In spite of indefiniteness of vegetal intersection points, it is clear that the stationary surface rings lean toward each other embracing a very obtuse angle.

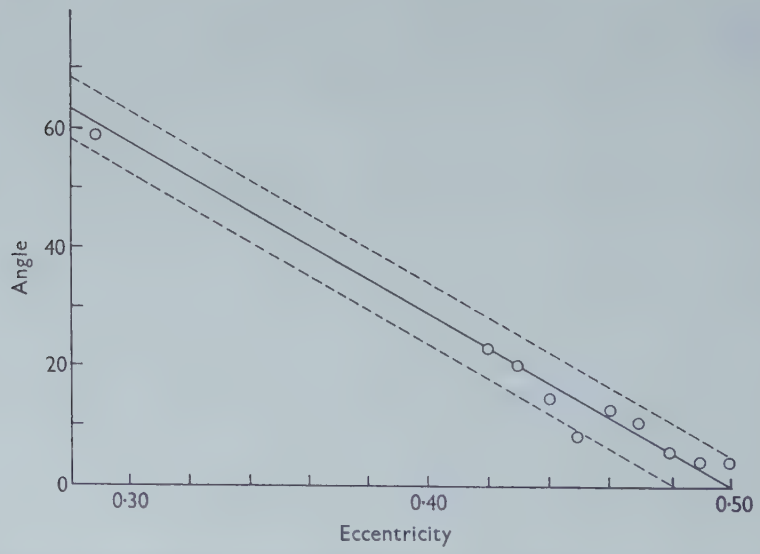

Fig. 6. Exact fit of the *Spirocodon* points on the *Astriclypeus* line. Extreme left point is average value of six *Spirocodon* cases. The group of points on the right are averages of *Astriclypeus* data as grouped by 0·01 unit of eccentricity.

Finally an attempt was made to extend the scheme to include the eggs of the medusan, *Spirocodon saltatrix*. The characteristic cleavage of medusae is considered by the author to be only an extreme case of heart-shaped division. Because of technical difficulties only a few data are available, but from these the following conclusions can be drawn. (1) The stationary lines become still more obscure. (2) It is, nevertheless, clear that the two lines incline toward each other, embracing a very obtuse angle between them. (3) Considering Fig. 3, together with Fig. 5, which illustrates medusan cleavage, the fact that as the cleavage process advances, the vegetal intersection points seem to be shifted toward higher levels suggests this as the cause of the indefiniteness of the vegetal intersection points. In the six observed cases of medusan cleavage, the average of the eccentricity is found to be 0·29 and that of the angle, 58·6°. These data are shown in Fig. 6 to fall on the same straight line as the averages for *Astriclypeus* eggs. This indicates that the same basic mechanism is involved in the cleavage of the two forms.

DISCUSSION

As was the case with regular cleavage (Ishizaka, 1958), the presence of a pair of stationary surface rings is extremely difficult to reconcile with existing theories of cell division, and the nature of these rings remains utterly unknown. However, the fact of a linear correlation between the obtuseness of the angle formed between the two planes of the stationary rings and the eccentricity of the spindle is beyond any doubt. In the author's previous analysis (Dan & Dan, 1947*a*, *b*), the conclusion was drawn that in the division of a cell whose spindle is eccentric in position, the two asters rotate in such a direction that their animal pole sides converge and their vegetal sides diverge. This is further manifested by the bending of the spindle so that it becomes convex toward the vegetal pole. The conversion of the two stationary rings toward the animal pole seen here must certainly be caused by the rotation of the internal asters. The same astral rotation may also have an indirect connexion with the V-shaped slanting of the astral loci.

SUMMARY

1. The eggs of the sand dollar, *Astriclypeus manni*, and the medusa, *Spirocodon saltatrix*, were used for the reason that they cleave in heart shape, the cleavage furrow appearing earlier at the animal than at the vegetal pole.

2. By the superposition of drawings showing contours and astral centres as well as the positions of carbon markers on the cell surface, the presence of a pair of stationary circular zones of the cortex can be demonstrated. These remain absolutely stationary through successive stages of cleavage, as was shown to be true of regularly cleaving sea-urchin eggs.

3. The two planes determined by this pair of stationary surface rings tilt toward each other on the animal pole side in linear proportion to the eccentricity of the mitotic spindle within the cell, and the loci of the astral centres tend to slant toward the animal pole.

4. The above phenomena can be explained by the previously proposed theory for heart-shaped cleavage; i.e. the primary cause of heart-shaped cleavage is the eccentric position of the spindle, which in turn causes the rotation of the asters and the bending of the spindle.

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A QUANTITATIVE DESCRIPTION OF PROTOPLASMIC MOVEMENT DURING CLEAVAGE IN THE SEA-URCHIN EGG

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Quantitative information on the protoplasmic movement during cleavage is one of the most important desiderata in the study of cell division. Spek (1918) described the protoplasmic streaming during cleavage and emphasized the importance of the surface tension in the cell division, giving attention to the similarity of this protoplasmic streaming to the streaming within an oil drop dividing as a result of local difference in surface tension. Dan and his collaborators (Dan, Yanagita & Sugiyama, 1937; Dan, Dan & Yanagita, 1938; Dan & Dan, 1940, 1942, 1947; Dan, 1943; Dan & Ono, 1954; Ishizaka, 1958) reported a series of experiments in which the cortical movement during cleavage was followed by measuring the movement of kaolin particles adhering to the cell surface. The results obtained by Spek and by Dan provide qualitative information on the protoplasmic movements during cleavage. However, from their data it is neither possible to make a quantitative analysis of the protoplasmic movement nor to infer the correlation between cortical and endoplasmic movements in detail. In the present work protoplasmic (both cortical and endoplasmic) movement during cleavage of the sea-urchin egg is described in a quantitative fashion, in order to provide basic data for the study of cell division.

The determination of the location of the motive force is an essential point for the study of cell division. Recently, cleavage without mitotic apparatus was reported in sea-urchin eggs by Swann & Mitchison (1953) and by the author (Hiramoto, 1956), which is favourable for the cortical activity theory. The present work is concerned with the protoplasmic movement during cleavage of the egg without mitotic apparatus, and seeks to compare the effect of the presence and absence of the mitotic apparatus on the distribution of stress within the dividing cell.

MATERIAL AND METHODS

As material the eggs of the heart-urchin, *Clypeaster japonicus* were used. Eggs were fertilized in the ordinary way and both the fertilization membranes and the hyaline layers were dissolved in an isotonic solution of urea. The eggs were then washed with fresh sea water and were cultured in a Syracuse dish until the amphiaster stage. Before experimentation, a drop of egg suspension was put on a slide, together with blocks of thin glass plate as supports, and a cover-slip was put over it. Because the

drop was surrounded with the blocks of glass plate and separated from them by an air gap, the eggs were not compressed by the cover-slip and the evaporation of the medium was minimized. When it was necessary to abolish evaporation completely, mineral oil was applied between the cover-slip and the blocks; but this was usually unnecessary. For the measurement of protoplasmic movement eggs were selected in which the spindle lay in the horizontal plane.

Determination of cortical movement. For the determination of cortical movement carbon particles were previously attached to the surface of the egg and the movement of the particles on the circumference of the largest optical section of the egg was traced by photographing them at appropriate time intervals.*

Photographs obtained from the same egg at successive stages of cleavage were projected on a sheet of graph paper;* the contours of the egg and the positions of the surface indicated by the particles in successive stages were traced; the tracings were then superimposed, using the spindle axis and the cleavage plane as reference, in the way described by Ishizaka (1958) (cf. Fig. 1).†

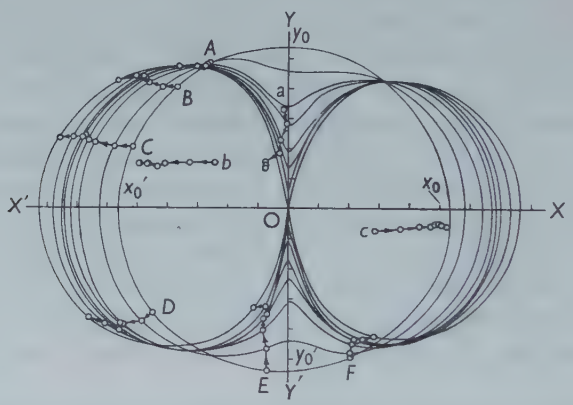

Fig. 1. Movement of particles adhering to the cell surface (*A*, *B*, *C*, *D*, *E*, *F*) and movement of endoplasmic granules (*a*, *b*, *c*) during cleavage of an egg of *Clypeaster japonicus*. The *x*-axis represents the spindle axis and the *y*-axis the cleavage plane. The smallest divisions on the co-ordinate axes are 10 μ.

Since only a few particles are found on the circumference of an egg at one time and their points of adherence to the cell surface are determined by chance, movement of only particular portions of the cortex is revealed by a single experiment. In consequence, data obtained from individual eggs are not directly comparable one with another because of individual differences as to (1) the size of the egg, (2) the stages of cleavage photographed, and (3) the positions on the cortex of the particles whose movements were traced.

* Distortion of the image by the optical system was almost negligible, since the distortion due to the objective lens was well compensated by a 'Periplan' eye-piece and that due to the projection lens could be eliminated by using an iris diaphragm.

† The spindle axis is represented by the *x*-axis, and the cleavage plane in the largest optical section is represented by the *y*-axis.

As shown in Fig. 1, *Clypeaster* eggs are not perfectly spherical even before the initiation of furrowing, and cleavage is not perfectly symmetrical with respect both to the spindle axis (x-axis) and to the cleavage plane (y-axis). Thus the circumference of the egg just before cleavage intersects the co-ordinate axes at different distances from the origin (O). Now, these distances are defined to be x_0, $-x_0'$, y_0 and $-y_0'$ (Fig. 1), x_0' and y_0' being negative. In order to convert the movement of a portion of the cortex, as obtained from the tracings of photographs, into normalized movement as for an egg having a radius of unity, x/x_0 (or x/x_0') and y/y_0 (or y/y_0') are taken as co-ordinates of the portion (x, y), whose movement was traced, where x_0 (or x_0') and y_0 (or y_0') have the same signs as x and y, respectively, i.e. both x/x_0 (or x/x_0') and y/y_0 (or y/y_0') are positive. For example, for normalized co-ordinates of point B in Fig. 1 just before cleavage ($-36{\cdot}8\mu$, $+40{\cdot}5\mu$),

$$x=0{\cdot}651\ (=-36{\cdot}8/-56{\cdot}5) \quad \text{and} \quad y=0{\cdot}751\ (=40{\cdot}5/53{\cdot}9)$$

are taken, since x_0' and y_0 are $-56{\cdot}5\mu$ and $+53{\cdot}9\mu$, respectively (cf. Fig. 2).

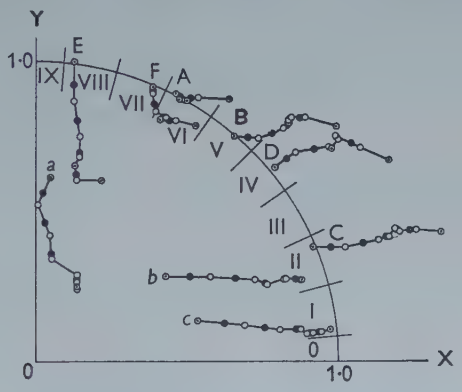

Fig. 2. Normalized movement of the surface particles and of the endoplasmic granules from Fig. 1. White circles indicate normalized positions. Black circles indicate normalized positions at reference stages which are determined by interpolation.

Since the stages of cleavage at which the egg was photographed are different in each egg, it is necessary to standardize the stages of cleavage in order to correlate the results obtained from different eggs. The stage of cleavage is defined by the percentage of the width of the furrow neck to the original egg diameter; e.g. the stage of cleavage is '100%' just before the beginning of furrowing, it is '0%' when the egg has just divided, and intermediate values are allotted to intermediate stages. In the present study, stages '100%', '90%', '70%', '50%', '30%', '15%' and '0%' are taken as reference stages. Positions of the cortical point at the reference stages are determined by interpolation (black circles in Fig. 2).

In order to obtain averaged movement of the cortex, experimental results are divided into ten groups according to topographical positions of the cortical points with reference to the cleavage plane in the following way (cf. Fig. 2). In the normalized graph, if the angle (θ), between the x-axis and the line connecting the origin (O) to the point which is moving, turns out to be larger than 5° and smaller

than 15° at stage '100%', such data are classified as group I. Cases in which θ is larger than 15° and smaller than 25° at stage '100%' are referred to group II. Similarly, group 0 ($5° > \theta$), group III ($35° > \theta > 25°$), group IV ($45° > \theta > 35°$), group V ($55° > \theta > 45°$), group VI ($65° > \theta > 55°$), group VII ($75° > \theta > 65°$), group VIII ($85° > \theta > 75°$) and group IX ($\theta > 85°$) are defined. The x-co-ordinates and the y-co-ordinates of all cortical points belonging to the same group at the same stage are averaged. The results are shown in Table 2 and in Fig. 4 (C_1, C_2, ..., C_8); in this figure the averaged positions in group 0 and in group IX are not shown, the averaged positions of the pole (C_p) and of the furrow (C_f) being indicated instead.

Determination of endoplasmic movement. In some eggs of *Clypeaster*, several granules within the cytoplasm are clearly distinguishable from other cytoplasmic granules by their larger size (about 1μ in diameter). The number of recognizable granules in the largest optical section of the egg differs in batches, up to ten in some batches and none in others. The movement of endoplasm could be determined using these granules as markers.

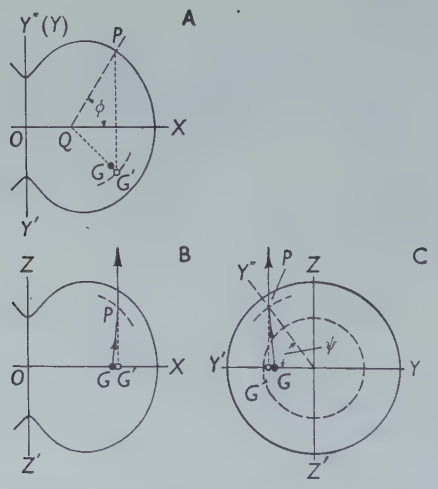

Fig. 3. A diagram indicating the refraction of light at the surface of a dividing sea-urchin egg. The spindle axis of the egg is taken as the x-axis (XX'), and cleavage plane is represented by the Y–Z plane. The optical axis of the microscope is the z-axis (ZZ'). The optical path is indicated by arrows in B and C. A: view from z-direction; the optical axis is perpendicular to the plane of the page; B, view from y-direction; C, view from x-direction. For further explanation, see text.

As is pointed out elsewhere (Hiramoto, 1957), since the light passing through egg protoplasm is refracted at the cell surface, the apparent positions of granules, as seen in the microscope, are not necessarily the true ones. The degree of the optical deviation from the true position can be calculated, if the refractive index of egg protoplasm and the geometrical form of the cell are given. The refractive index of egg protoplasm has been obtained (the first method in Hiramoto, 1957) and was found to be 1·385 using eggs shortly before cleavage.*

* Refractive index of sea water: 1·3383 (25° C.).

The correction of the position of the granule is made assuming that the refractive index remains unchanged during cleavage and that the egg has an axial symmetry. In Fig. 3 true and apparent positions of a granule in the largest optical section are shown (G and G'). The determination of the exact position of the granule by ray tracing is troublesome in procedure since the geometrical form of the egg is not simple. However, the true position of the granule (G) is approximately obtained by tracing the axial ray, since the maximal deviation of the position thus calculated is only of the order of 1μ and only rays which are within 30° (in sea water) from the optical axis enter the objective lens (40×, N.A.: 0·65).

P is the point where the cell surface is intersected by the line passing through G' parallel with the optical axis (ZZ' in Fig. 3B, C, which is perpendicular to the page in Fig. 3A); Q is the point where the x-axis is intersected by the line normal to the cell surface at P. The corrected position of the granule is $[x'-k(x'-q), y'-k'y']$, where q is x-co-ordinate of Q, and x' and y' are respectively the x- and y-co-ordinates of G'. k is a constant which changes in accordance with the angle (ϕ) between the normal PQ and x-axis and k' is another constant which varies with the angle (ψ) between the X–Y'' plane (including the x-axis and point P) and X–Y plane (cf. Table 1). Usually a value of 1/30 can be used as k or k', the error resulting from this approximation being negligible.

Table 1. *k- (or k'-) values for various φ- (or ψ-) values*

ϕ (or ψ)	90°	80°	70°	60°	50°	40°	30°	20°	10°
k (or k')	0·035	0·034	0·034	0·034	0·034	0·033	0·032	0·031	0·025

Typical movement of endoplasmic granules in the largest optical section of an egg is shown in Fig. 1. The movement of the granules is normalized in the same way as in the analysis of the cortical movement by taking x/x_0 (or x/x_0') and y/y_0 (or y/y_0'). For example, the normalized position of the granule b (Fig. 1) at the initial spherical stage is (0·428, 0·282) (Fig. 2), as its position is $(-24{\cdot}2\mu, +15{\cdot}2\mu)$ and x_0' and y_0 are $-56{\cdot}5\mu$ and $+53{\cdot}9\mu$ respectively. The positions of the granule at the reference stages (100, 90, 70, 50, 30, 15 and 0%) are obtained by interpolation (black circles in Fig. 2).

Normalized positions of the granules at stage 100% are indicated by white circles in Fig. 4A. These points are obtained from seventeen selected eggs dividing with considerable regularity. Regular cross-lines at stage 100% (Fig. 4A) indicate different strata in the endoplasm, the distortion of which in Fig. 4B–G shows general movement within the cell.

Colchicine treatment. Cortical and endoplasmic movements during cleavage were similarly obtained in eggs treated with colchicine. The eggs deprived of both the fertilization membranes and the hyaline layers were treated with 3×10^{-3} M or 5×10^{-3} M colchicine shortly before cleavage, and the determination of protoplasmic movement was made after the disappearance of the mitotic apparatus.

RESULTS

I. *Normal egg*

In a diagram such as Fig. 1, in which contours of an egg at successive stages of cleavage are so superimposed that they share the spindle axis and cleavage plane, cortical movement shows the following characteristic features. Roughly speaking, the furrow region of the cortex moves toward the spindle axis parallel with the cleavage plane throughout the cleavage (*E*), the polar region of the cortex moves in the polar direction parallel with the spindle axis (*B*, *C*, *D*), and the borders between these regions scarcely move (*A*, *F*) during cleavage. The presence of such borders which do not move during cleavage was discovered in other species of sea urchin by Ishizaka (1958) who named them 'stationary surface rings'.

Cytoplasmic granules in the region close to the cleavage plane move toward the spindle axis but away from the cleavage plane during cleavage (cf. *a* in Fig. 1); the granules in the region close to the spindle axis move in the polar direction (*b*, *c* in Fig. 1); the granules in the peripheral region of the cell move together with the cortex. Both cortical and endoplasmic movements are symmetrical with respect to both the spindle axis and the cleavage plane. These results are more clearly and quantitatively indicated in the averaged results in Fig. 4.*

Since the movement of every point in the egg can either be computed from Table 2 or can be graphically estimated from Fig. 4, various conditions governing protoplasmic movement during cleavage can be anticipated as follows.

Constancy of the cell volume during cleavage. The total volume of the egg is calculated as the revolution about the spindle axis of the shape of the egg in the largest optical section shown in Fig. 4. The volume of the egg equals $2\pi\int_0^{x_p} y^2 dx$, in which y is the y-co-ordinate of the cell surface and x_p is the x-co-ordinate of the pole (C_p). The values are calculated using Simpson's formula. The result is shown in Table 3. Considering the fact that an error of $0{\cdot}4\mu$ in the egg diameter would result in a volume error of more than 1%, the constancy of the volume is surprising.

Linear and areal changes of the cell surface during cleavage. Dan and his collaborators (Dan *et al.* 1937; Dan *et al.* 1938; Dan & Dan, 1940; Dan & Ono, 1954) described regional differences in the degree of expansion of the circumference of sea-urchin eggs during cleavage. Similar results, shown in Fig. 5, are obtained by measuring the linear change of the circumference of the egg indicated in Fig. 4. Polar region, subpolar region, subfurrow region and furrow region by the definition of Dan *et al.* correspond to regions between C_p and C_4, C_4 and C_6, C_6 and C_8, and C_8 and C_f in Fig. 4, respectively. The contour along the circumference of the egg is

* As is mentioned elsewhere (Hiramoto, 1957), there is a cortex at the surface of sea-urchin egg which differs in physical properties from the endoplasm. Therefore some discontinuity in the cytoplasmic movement is to be expected at the boundary between the cortex and the endoplasm because of the high rigidity of the cortex. Although this is not indicated in the figure, since the precise determination of displacement within such a narrow space was impossible, it does not seem that greater precision would invalidate the argument in the present paper.

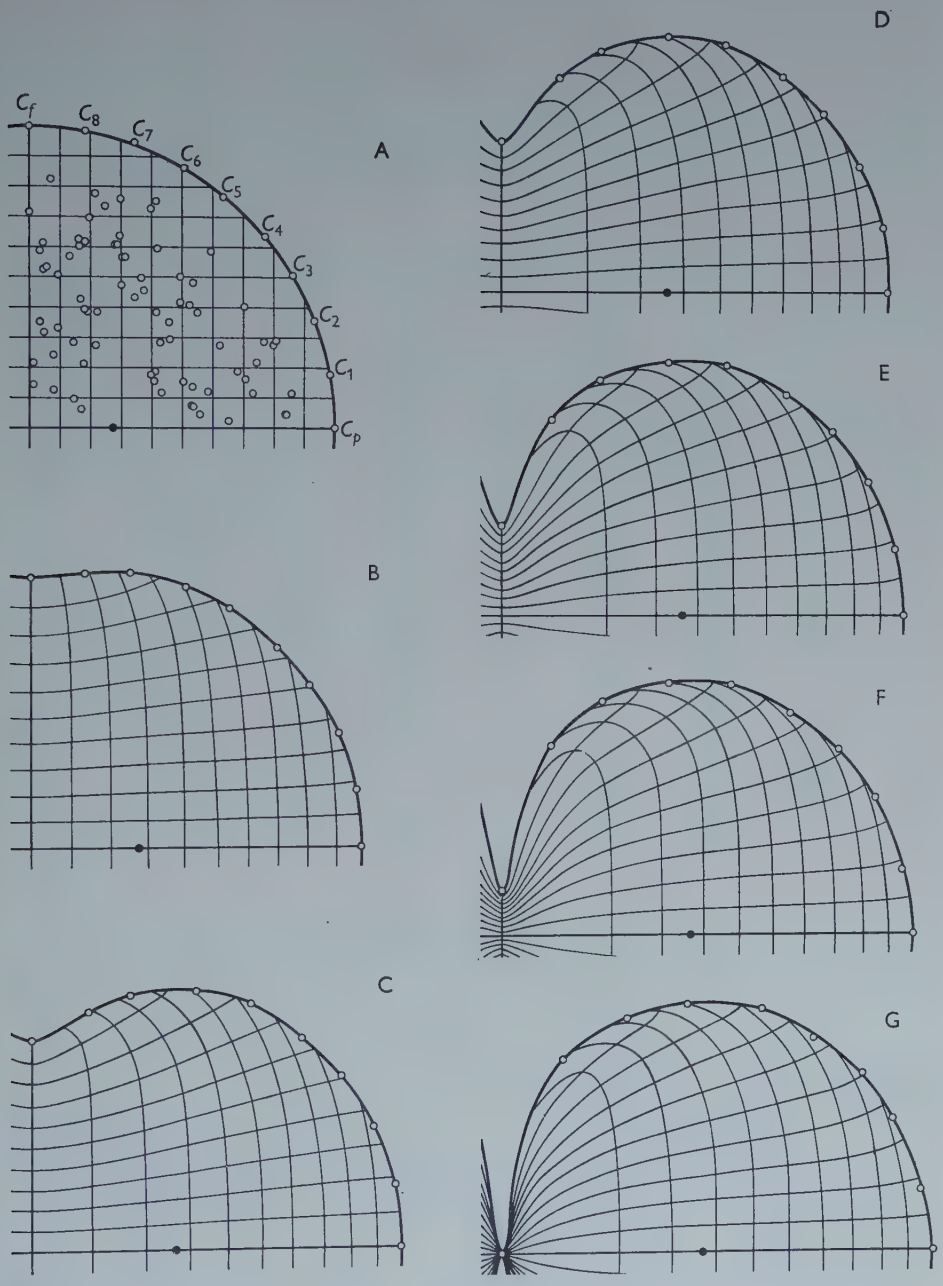

Fig. 4. Diagram indicating protoplasmic movement during cleavage of *Clypeaster* egg. A quarter of the egg is shown. A, stage 100 %; B, stage 90 %; C, stage 70 %; D, stage 50 %; E, stage 30 %; F, stage 15 %; G, stage 0 %. Cross-lines within the egg indicate different strata of the endoplasm in the largest optical section of the egg. Movements of white circles on the cell surface in A (C_p, C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_f) can be followed in the other figures. Black circles indicate positions of the astral centre.

determined by assuming that it consists of series of circular arcs.* It will be seen from Fig. 5 that the general characteristics of the linear changes of the surface along the circumference coincide with those found by Dan *et al.* except for small quantitative differences, some of which may be attributable to the difference in material.

Changes in area of the surface during cleavage are shown in Fig. 7A. The surface area is calculated by assuming that the blastomeres consist of series of zones and

Table 2. *Averaged cortical movement of the sea-urchin egg during cleavage*

Stage		100%	90%	70%	50%	30%	15%	0%	No. of points averaged
C_p	x	1·000	1·080	1·208	1·288	1·344	1·374	1·438	36
	y	0·000	0·000	0·000	0·000	0·000	0·000	0·000	
C_1	x	0·984	1·067	1·190	1·274	1·313	1·337	1·398	13
	y	0·178	0·189	0·207	0·217	0·221	0·215	0·201	
C_2	x	0·933	1·008	1·120	1·193	1·224	1·249	1·303	17
	y	0·356	0·379	0·401	0·421	0·447	0·454	0·440	
C_3	x	0·861	0·912	1·013	1·073	1·103	1·126	1·203	17
	y	0·507	0·538	0·572	0·599	0·616	0·620	0·593	
C_4	x	0·771	0·807	0·886	0·938	0·950	0·962	1·037	19
	y	0·635	0·663	0·700	0·721	0·739	0·741	0·714	
C_5	x	0·634	0·650	0·719	0·747	0·751	0·767	0·867	10
	y	0·766	0·796	0·816	0·828	0·837	0·838	0·813	
C_6	x	0·507	0·505	0·537	0·554	0·555	0·558	0·619	20
	y	0·861	0·867	0·860	0·853	0·847	0·843	0·830	
C_7	x	0·344	0·325	0·321	0·332	0·326	0·338	0·417	12
	y	0·947	0·915	0·848	0·803	0·788	0·782	0·783	
C_8	x	0·183	0·177	0·185	0·192	0·168	0·165	0·202	15
	y	0·985	0·912	0·794	0·714	0·654	0·635	0·648	
C_f	x	0·000	0·000	0·000	0·000	0·000	0·000	0·000	
	y	1·000	0·900	0·700	0·500	0·300	0·150	0·000	

Table 3. *Volume of dividing sea-urchin egg*

Stage	100%	90%	70%	50%	30%	15%	0%
Volume (%)	100·0	100·0	99·9	99·5	99·8	100·6	100·3

segments of spheres following the method of Dan & Ono (1954), although the procedure of calculation is different. In Fig. 6B, the area of curved surface of the zone is $2\pi dy$, in which d is the length of the chord (LN) and y is the distance between the axis of rotation and the mid-point (M) of the arc. The total area is the sum of these areas ($2\pi\Sigma dy$). In the case of the segment of a sphere the surface area can be calculated in the same way. This method of calculation is as simple as that of Motomura (1940), which is less accurate than the present one because his calcula-

* Between length of the arc (l in Fig. 6A), length of the chord (d), height of the bow shape (x), and angle α, the following relations hold:

$$l/d = \alpha/(2 \sin \tfrac{1}{2}\alpha), \quad x/d = \tfrac{1}{2} \tan \tfrac{1}{4}\alpha.$$

Therefore, l is directly obtained using a graph of $\tan \frac{1}{4}\alpha$ plotted against $\alpha/\sin \frac{1}{2}\alpha$.

tion is made under an assumption that the blastomeres consist of series of trapezoids, and is as accurate as that of Dan & Ono (1954), which is somewhat troublesome in procedure because, to obtain the area of the surface, a circle which would circumscribe the curved surface of the cell must be found by successive approximation. Moreover, the present method is applicable with exactitude when the cell surface is a part of a cone (Fig. 6C), and is applicable with approximation unless the curvature

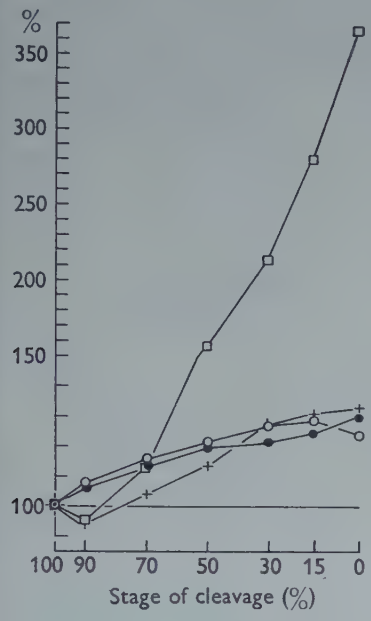

Fig. 5

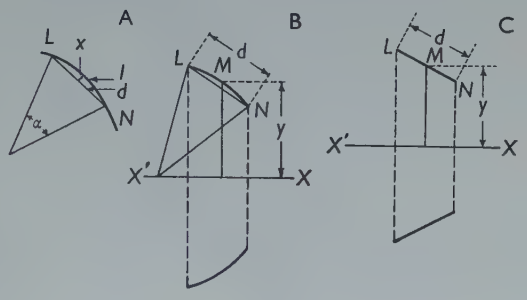

Fig. 6

Fig. 5. Linear elongation or shrinkage of the cell surface during cleavage. Curves are constructed by measuring sections of the circumference between two adjacent white circles shown in Fig. 4. ○, length between C_2 and C_4 (polar region); ●, length between C_4 and C_6 (subpolar region); +, length between C_6 and C_8 (subfurrow region); □, length between C_8 and C_f (furrow region).

Fig. 6. Diagram illustrating the methods of calculation of the length and the area of curved surface. A: the length of the arc (LN) is estimated from the relation between $\tan \frac{1}{4}\alpha$ and $\alpha/\sin \frac{1}{2}\alpha$, because $l/d = \alpha/(2 \sin \frac{1}{2}\alpha)$ and $x/d = \frac{1}{2} \tan \frac{1}{4}\alpha$, where l is the length of the arc (LN), d is the length of the chord (LN), and x is the height of the bow shape. B: the area of the surface of a zone of a sphere having the axis (XX') is $2\pi dy$. The largest optical section of the zone is indicated by heavy lines, where d is the length of the chord (LN) and y is the distance of the mid-point (M) of the arc from the axis (XX'). C: the area of the surface of a cone, the cross-section of which is indicated by heavy lines, is also $2\pi dy$, where d is the length of line (LN) and y is the distance of its mid-point (M) from the axis of the cone (XX').

of the cell surface is too large. As is shown in Fig. 7A, the result agrees in general with that of Dan & Ono (1954).

Fig. 7B indicates the areas occupied by each of the four regions as percentages of the total surface area at various stages of cleavage. In the early stage of cleavage (100–70%), furrow and subfurrow surfaces contract, whereas polar and subpolar surfaces expand. In the middle stage (70–15%), the percentage of area occupied

by each region is almost unchanged although total area increases. In the late stage (15–0%), furrow surface expands, polar surface contracts, and subfurrow and subpolar surfaces retain their percentages of the total surface area almost unchanged. Areal change in the late stage mentioned above implies the initiation of a large expansion of the furrow membrane accompanying the contraction of the polar membrane which has been pointed out by Dan *et al.* (Dan *et al.* 1938; Dan & Dan, 1940; Dan, 1954) as a characteristic feature of the post-cleavage period.

General characteristics of endoplasmic movement. Endoplasmic movement indicated in Fig. 4 is qualitatively similar to that reported by Spek (1918). It will be noted in this figure that although the vertical lines within the endoplasm in A are not so much deformed in the central zone of the egg during the course of cleavage, they are much deformed in the peripheral zone.

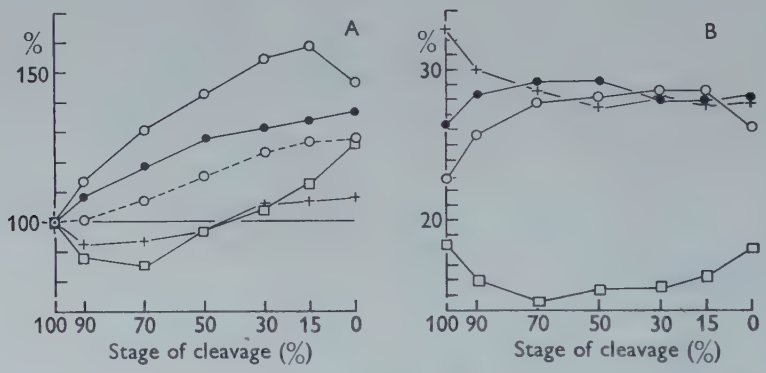

Fig. 7. Areal change of the cell surface during cleavage. Curves are constructed by measuring the surface of dividing egg shown in Fig. 4. A: percentage changes, taking the initial state as 100. Broken line indicates change of the total surface area. B: changes in area of individual regions as percentages of the total surface area. Definition of symbols is the same as in Fig. 5.

This fact is more clearly shown by the movement of endoplasm through the stationary surface ring (Fig. 8). In Fig. 8A, the y-axis represents the plane of the stationary surface ring in the largest optical section of the egg.* This figure illustrates the endoplasmic movement through the stationary surface ring by indicating where endoplasmic elements, constituting the plane of the stationary surface ring at the stage 70%, were at stages 90 and 100% and where they will be at future stages. To avoid overlapping of curves, the scale of the x-axis of Fig. 8A is magnified five times in Fig. 8B. It is clear that in the central part the lines are approximately parallel, while in the peripheral zone they converge rather steeply toward the y-axis as much-deformed parabolas. This fact may suggest the presence of some gelated structures such as the spindle and the asters which impede the free movement of

* As is shown in Fig. 4, every point on the cell surface changes its position in the $x-y$ diagram in a strict sense. Therefore, for convenience, the part of the cell surface which is in position (0·550, 0·831) in Fig. 4A (stage 100%) is taken as 'stationary surface ring' because it is almost motionless. This part, of course, moves during cleavage, e.g. it is in (0·600, 0·855) at stage 70% (Fig. 4C). The y-axis in Fig. 8 is determined in such a manner that it passes the above-mentioned part, namely (0·550, 0·831) at stage 100% and (0·600, 0·855) at stage 70%.

protoplasm in the central zone of the cell, because it is expected that the endoplasmic displacement curves ought to approach perfect parabolas if the endoplasm is composed of a homogeneous liquid whose movement during cleavage is caused solely by the deformation of the cortex. Kamiya (1950) reported a velocity distribution curve of similar shape in the intracapillary flow of the protoplasm of myxomycete plasmodium.

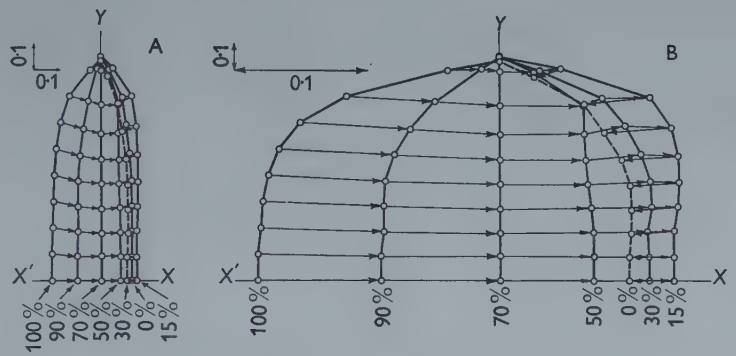

Fig. 8. Displacement of endoplasm within the plane of the stationary surface ring of the dividing sea-urchin egg. Curves are constructed from the data in Fig. 4. Though the x-axis (XX') of the figure is the same as the abscissa of Fig. 4, the y-axis here is taken to indicate the plane of the stationary surface ring in the largest optical section of the egg. Circles connected by heavy lines represent loci of endoplasmic elements which constitute the plane of the stationary surface ring at the stage 70%. The scale of the x-axis in A is magnified five times in B.

Backward movement of endoplasm during the period between stages 15 and 0% (Fig. 8) may be due to the initiation of the expansion of the furrow membrane mentioned above.

Change in shape and length of astral rays during cleavage. Black circles in Fig. 4 indicate the averaged positions of the astral centre. It will be noted that although the absolute position of the astral centre changes during cleavage, its position relative to the surrounding protoplasm is unaffected. In other words, the astral centre moves together with surrounding protoplasm, i.e. no slipping occurs between them.

The changes in shape of the astral rays can be estimated graphically by assuming that the individual parts of the astral rays move together with surrounding protoplasm over their entire lengths. In Fig. 9, ten astral rays designated A_p, A_1, A_2, A_3, A_4, A_5, A_6, A_7, A_8 and A_f are represented, which are attached to the cortex at points C_p, C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8 and C_f (Fig. 4), respectively. In this figure, the peripheral parts of the astral rays indicated by broken lines are hardly visible in living material. Although it is unlikely that the astral rays in the peripheral zone of the egg have rigidity, especially in the late stage of cleavage, it is probable that the material of the rays may remain in the positions indicated by broken lines. It will be seen that the astral rays, which are straight at stage 100%, bend during cleavage. The bending of astral rays during cleavage has been observed by many investigators (cf. Dan, 1943).

Fig. 10 indicates the changes in length of these astral rays during cleavage, as obtained from Fig. 9. Astral rays attached to the furrow region of the cortex (A_f, A_8) shorten in the early stage of cleavage. If the furrowing results from the mechanical pull of the astral rays attached to

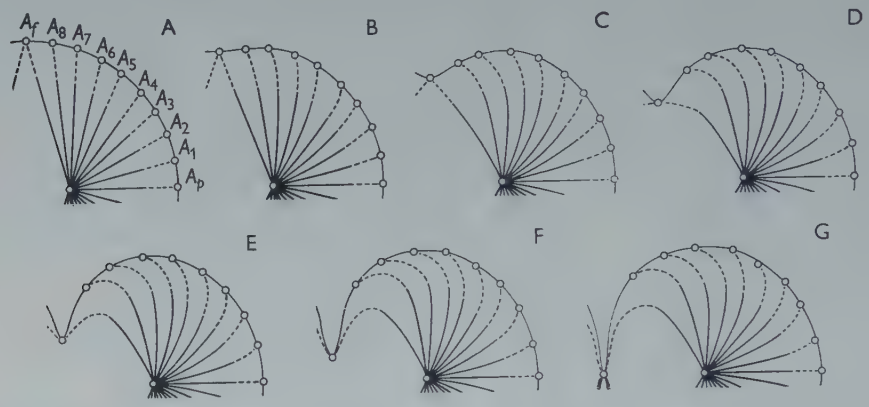

Fig. 9. Changes in shape of the astral rays during cleavage. The shapes of the astral rays are estimated graphically from the protoplasmic movements indicated in Fig. 4. A_p, A_1, A_2, A_3, A_4, A_5, A_6, A_7, A_8 and A_f are hypothetical astral rays attached to the cortex at points C_p, C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8 and C_f (Fig. 4), respectively. Peripheral parts of rays indicated by broken lines are hardly seen in living materials.

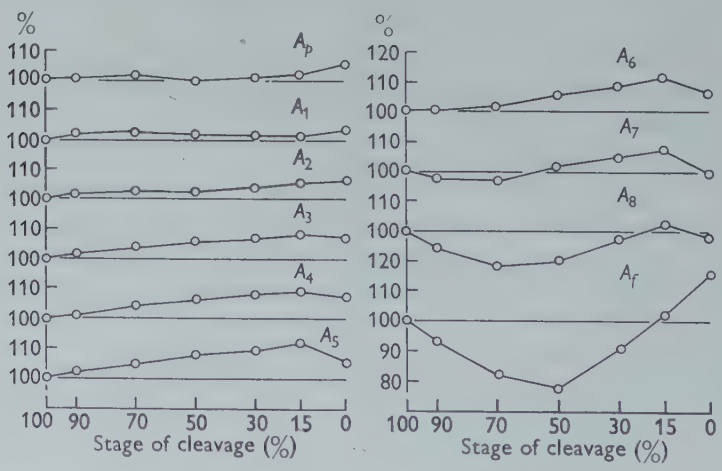

Fig. 10. Changes in length of astral rays during cleavage. A_p, A_1, A_2, A_3, A_4, A_5, A_6, A_7, A_8 and A_f are the hypothetical astral rays indicated in Fig. 9.

the furrow cortex must be stretched. On the other hand, if the furrowing results from the active constriction of the furrow cortex, the astral rays must be shortened by compression. The result shown in Fig. 10 is favourable to the latter interpretation, i.e. the contracting-ring theory is preferable to the radiate-aster theory (Dan, 1943) in this connexion.

Behaviour of an injected oil drop. The normalized endoplasmic movement of Fig. 4 was checked by observing the movement of paraffin oil injected into eggs. The behaviour of the successfully injected drop was roughly the same as that of the endoplasmic granules mentioned above.* In some eggs, however, slight deviation of the movement of the oil drop was observed from that expected from Fig. 4. This deviation may be due to imperfect recovery of the cell from the deformation at the time of micro-injection. Simultaneous observation of the oil drop and of endoplasmic granules in the same position within the same egg did not reveal any difference in the movements of these two kinds of marker.

II. *Egg without mitotic apparatus*

Averaged protoplasmic movement of the colchicine-treated egg is shown in Fig. 11. Although the spindle and the peripheral parts of the asters disappear after treatment with colchicine, the central parts of the asters do not completely disappear; they remain as two bright areas within the cell even when the drug is sufficiently concentrated to inhibit cleavage. Black circles in Fig. 11 indicate the averaged positions of the centre of this bright area. It will be seen that this point moves together with the surrounding protoplasm. The difference in position between the bright spot in colchicine and the astral centre in the normal egg (black circles in Fig. 4) may be due to the rearrangement of endoplasm resulting from disintegration of the mitotic apparatus.

Figs. 12 and 13 indicate linear and areal changes of the cell surface during cleavage of the colchicine-treated egg. The results are similar to those for the normal egg except that the changes are slightly more exaggerated in the colchicine-treated egg than in the normal one.

In contrast to the cortical movement, the endoplasmic movement is much affected by treatment with colchicine. The displacement of endoplasm in the central zone of the cell is much greater in the colchicine-treated egg than in the normal egg. Vertical lines in Fig. 4 appear as curved (convex) toward the pole in Fig. 11. Fig. 14, which corresponds to Fig. 8B in the normal egg, brings out this fact more clearly. The displacement curves of endoplasm through the stationary surface ring† are almost simple parabolas with their axes coinciding with the spindle axis. Since the protoplasmic viscosity decreases after colchicine treatment (Swann & Mitchison, 1953), it seems likely that the impediment to the movement of the endoplasm is absent in the colchicine-treated egg. From these facts and from the argument in the preceding section it is suggested that the displacement curves indicated in Fig. 14 are interpreted as passive flow of endoplasm which has become solated by the treatment with colchicine.

* In some experiments, the injected drop was gradually pushed out through the path of the insertion of the pipette. This may be due to unsuccessful impalement by the micro-pipette, as pointed out by Tyler, Monroy, Kao & Grundfest (1956).

† The position on the cell surface which is at (0·530, 0·850) at stage 100 % and is at (0·550, 0·856) at stage 70 % in Fig. 11.

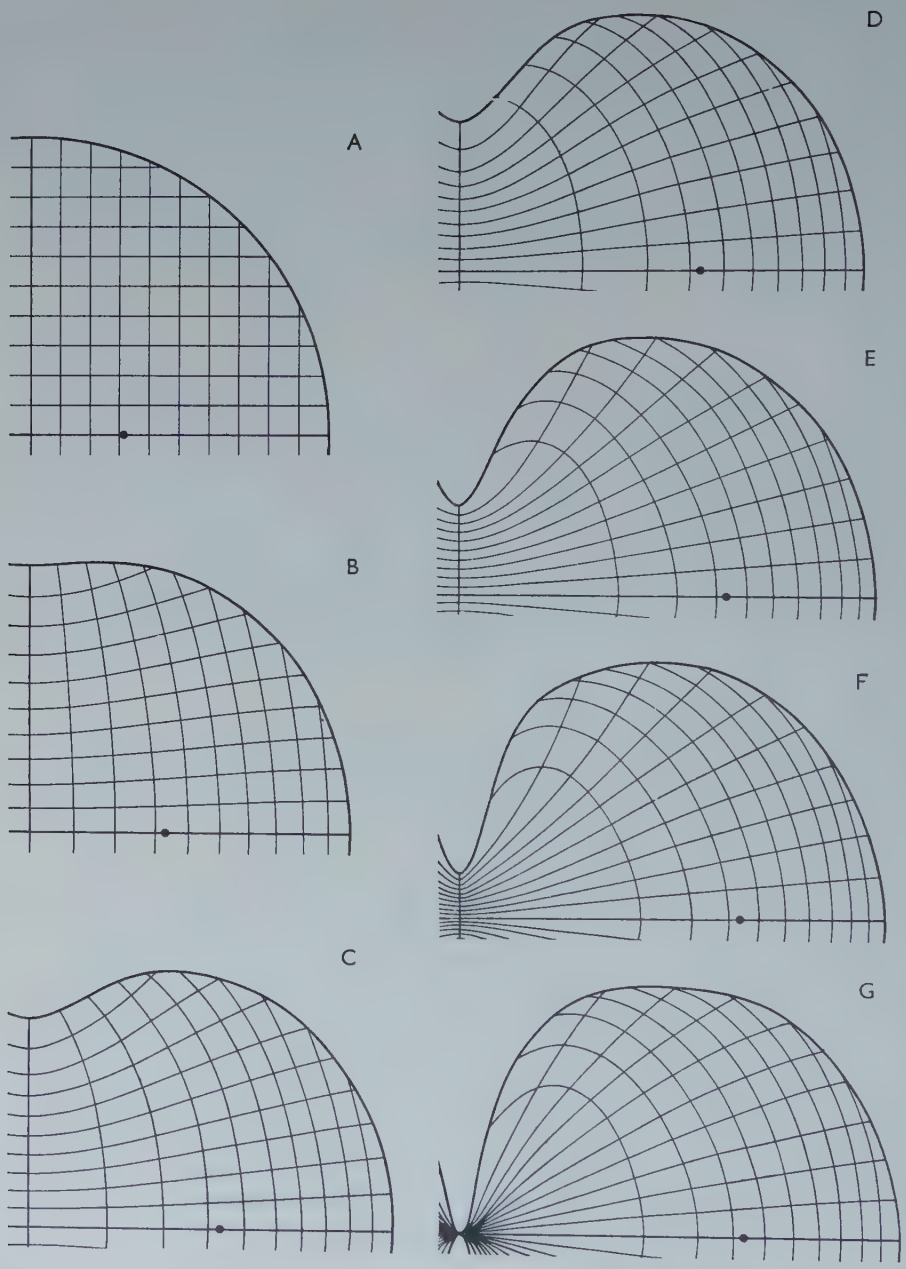

Fig. 11. Diagram indicating the protoplasmic movement during cleavage of the colchicine-treated egg. A, stage 100 %; B, stage 90 %; C, stage 70 %; D, stage 50 %; E, stage 30 %; F, stage 15 %; G, stage 0 %. Cross-lines within the egg indicate different strata of the endoplasm in the largest optical section of the egg. Black circles indicate positions of the remnant of the astral centre.

An attempt was made to trace the protoplasmic movement during cleavage in eggs from which the spindles had been removed by sucking them into a micro-pipette (Hiramoto, 1956). However, since the genuine movement of protoplasm

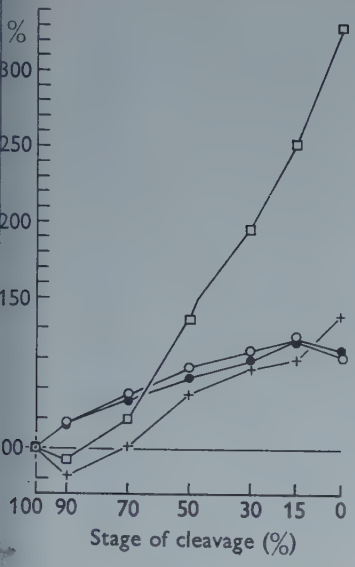

Fig. 12

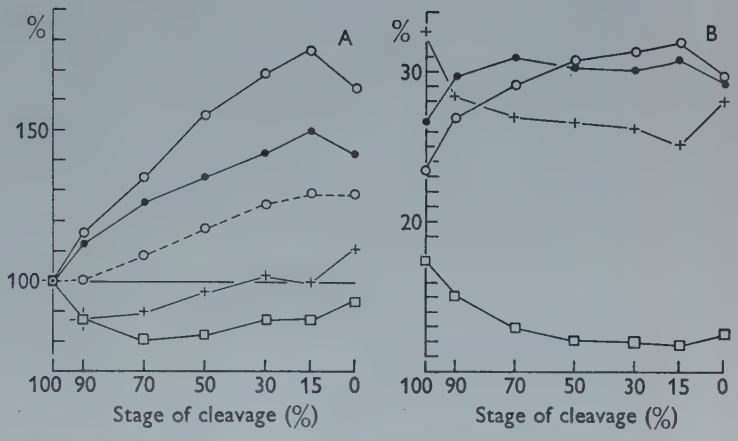

Fig. 13

Fig. 12. Linear elongation or shrinkage of the cell surface during cleavage of the colchicine-treated egg. Curves are constructed from Fig. 11. ○, polar region; ●, subpolar region; +, subfurrow region; □, furrow region.

Fig. 13. Areal change of the cell surface during cleavage of the colchicine-treated egg. A: percentage changes taking the initial state as 100. Broken line indicates change in total surface area. B: changes in area of individual regions as percentages of the total surface area. Definition of symbols is the same as in Fig. 12.

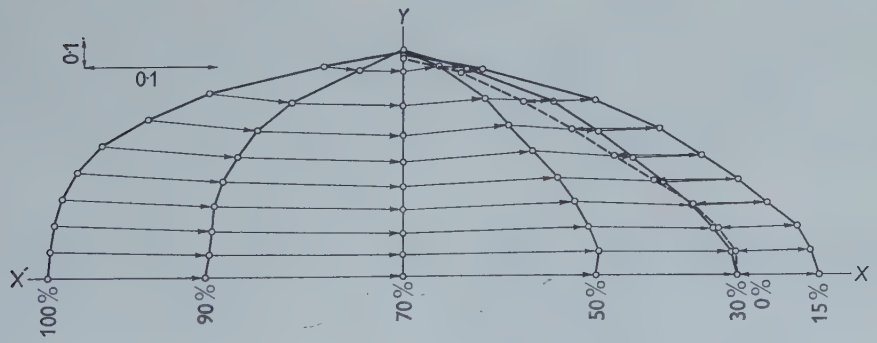

Fig. 14. Displacement of endoplasm through the plane of the stationary surface ring of the dividing colchicine-treated egg. The x-axis (XX') corresponds to the abscissa in Fig. 11. The y-axis indicates the plane of the stationary surface ring. Circles connected by heavy lines indicate the changes in position of endoplasmic elements constituting the plane of the stationary surface ring at the stage 70%. The scale of the abscissa is magnified five times.

by cell division overlaps with the protoplasmic rearrangement resulting from the recovery process from strain set up during the operation, it was impossible to obtain definite information.

DISCUSSION

In this study, cortical movement during cleavage has been described by using particles attached to the surface of the egg as markers. The movement of particles seems to represent the cortical movement of the egg in so far as no slipping occurs between the cortical cytoplasm and particles adhering to the cell surface (cf. Dan & Dan, 1940). Endoplasmic movement has been determined by tracing the movement of granules within the endoplasm. The movement of these granules represents the movement of the protoplasm in which they are suspended, because they move together with an injected oil drop whose movement seems to be entirely passive.*

Whether the motive force of cell division is located in the cortex or in endoplasmic structures such as the spindle and the asters is one of the most important problems concerning the mechanism of cell division. As is mentioned above, the shortening of astral rays attached to the furrow cortex in the early stage of cleavage suggests the active movement of the cortex. According to Dan (1943), the distance between the astral centre and the furrow surface (namely, the length of astral ray) is kept unchanged during early stages of cleavage in *Hemicentrotus* (*Strongylocentrotus*) and *Mespilia* eggs (cf. figs. 6 and 7 in his paper). In eggs of these species the shortening of the astral rays in the furrow region may be too small to be ascertained in early stages.

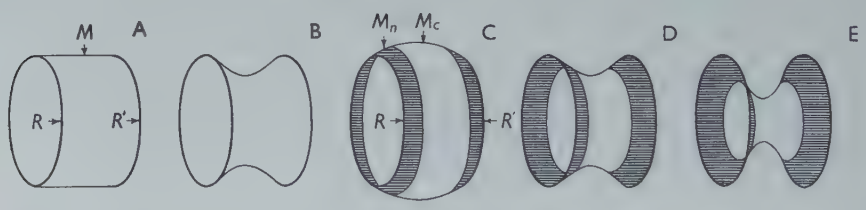

Fig. 15. Supposed changes in the shape of a membrane spanning the space between two immovable rings (a model imitating the behaviour of the furrow and the subfurrow regions of the dividing sea-urchin egg bordered on both sides by its stationary surface rings). A, B: uniformly contracting membrane (M) at rest (A) and in contraction (B). C, D, E: a membrane composed of contractile (M_c) and non-contractile (M_n) parts at rest (C) and in the process of contraction (D, E). R, R': immovable rings.

The three-dimensional contraction of the furrow surface cannot necessarily be expected from the contracting-ring (band) theory, because contraction of the furrow surface cannot take place freely since the volume of the cell is required to be kept unchanged. Now we shall imagine a model which is composed of two rigid rings of the same size lying in planes perpendicular to their common axis, like two wheels

* Iida (1942) found cytoplasmic granules stainable with neutral red in *Mespilia* and *Clypeaster* eggs which made peculiar movements, but they are different in kind from the granules in the present work.

on a shaft (*R* and *R'* in Fig. 15A) and a membrane connecting these two rings (*M*). The two wheels represent a pair of stationary surface rings. If this membrane shrinks in area while the distance between the rings is kept unchanged, the membrane must change its shape as shown in Fig. 15B: the area of the membrane decreases while the contour in the lateral view increases. If the surface bulges out in the lateral view as shown in Fig. 15C, and if it is further assumed that the contractile region is restricted to the middle part of the membrane (M_c) while the remaining parts are non-contractile (M_n), then the shape of the membrane, when M_c is made to contract, must be identical with the shape in Fig. 15B. In this case, the contour line of the active region in the lateral view will decrease when the contraction is small (Fig. 15D), but it will increase when the contraction is large (Fig. 15E), while its area decreases steadily regardless of the degree of contraction, and the non-contractile membrane (M_n) expands both in length and in area. The situation in dividing eggs is similar to that in the latter case. The behaviour of the surface of the furrow and subfurrow regions of the cortex in the early stages of cleavage can be imitated by the above model.*

'Contraction of the furrow cortex' in the present paper means the development of a contractility of the furrow cortex higher than that of other regions, but the other regions may also change their mechanical properties during cleavage (cf. Mitchison & Swann, 1955).

Increase in surface area in the furrow region during the late stages of cleavage (after stage 70%, Fig. 7A, B) must be explained by assuming that the contractility of the furrow cortex is greater in the direction parallel to the equator ('latitude') than in the direction along the lines joining pole to pole ('longitude').

Abrupt increase in area of the furrow surface in the late stage of cleavage (after stage 15%) accompanying the contraction of the polar surface has been pointed out in the present experiment. The author believes that the active phase of cell division almost comes to an end in the stage 15%, and subsequent separation of blastomeres can be brought about by their surface forces without further contraction of the furrow surface, in the same manner as the separation of an elongated liquid drop into two.

SUMMARY

1. Protoplasmic movements during cleavage in the eggs of the heart-urchin *Clypeaster japonicus* have been followed by tracing the movements of cytoplasmic granules and of carbon particles adhering to the surface.

2. These movements are quantitatively described in normal eggs and in eggs whose mitotic apparatus has been destroyed by colchicine.

3. The results obtained are qualitatively similar to those obtained by Spek and by Dan and his collaborators.

* An initial shrinkage phase in linear change is observed in the subfurrow region as well as in the furrow region in the present experiment (Fig. 5), while it is observed only in the furrow region in the experiments of Dan and his collaborators. This discrepancy may be due to the difference in the breadth of the contracting band in these two cases.

4. Endoplasmic movement and changes in the length and shape of the astral rays are readily explained by the contracting-ring (band) theory.

5. The location of the motive force of cell division is discussed.

The author acknowledges his indebtedness to Prof. H. Kinosita and Prof. K. Dan for their valuable criticisms and kind help in the preparation of the manuscript. He also thanks Prof. J. Koana for information concerning the optical problems.

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IONIC FLUXES IN *ARTEMIA SALINA* (L.)

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INTRODUCTION

In previous papers (Croghan, 1958*a*, *b*, *c*, *d*) various aspects of the osmotic and ionic regulation of *Artemia salina* have been described. It is also of interest to investigate the dynamic aspects of the ionic fluxes in *Artemia* using radio-active isotopes. This paper contains the results of these studies.

THEORETICAL CONSIDERATIONS

In an animal maintaining its ionic composition in a steady state the total flux of any ion is the same in both directions, the influx from the medium being balanced by an identical efflux. This flux can be expressed as mM. of ion entering (or leaving)/l. of haemolymph/hr.

At zero time a small amount of a radio-active ion is added to the medium. The amount added is so small compared with the amount of inactive ion already present that the steady state is unaffected. The haemolymph activity should rise and reach an equilibrium value asymptotically (cf. Fig. 1). The slope at any point on such an exchange curve is clearly

$$\frac{dy}{dt}=\frac{mx}{c_0}-\frac{my}{c_i},$$

where x is the activity (defined as the count rate for a unit volume under standard conditions) of the medium and y the activity in the haemolymph, c_0 is the concentration (mM./l.) of the ion in the medium and c_i the concentration in the haemolymph, and m is the total flux (mM./l. haemolymph/hr.) of the ion.

Integrating

$$y=\frac{c_i x}{c_0}(1-e^{-mt/c_i}).$$

Now $c_i x/c_0=z$ (the activity of the haemolymph when the exchange is complete). Substituting and solving

$$mt=c_i \log_e [z/(z-y)].$$

On plotting $2{\cdot}3c_i \log_{10} [z/(z-y)]$ against t a straight line should be obtained, the slope of which is the total ion influx (m). If the course of the exchange is not followed to effectively infinite time, z can be calculated from $c_i x/c_0$.

Similar information can also be obtained from the converse type of experiment. The animal is soaked in the radio-active medium, and the rate of loss of the active

ion is then studied in an inactive, but otherwise similar, medium of effectively infinite volume. The rate of fall of haemolymph activity is clearly

$$\frac{dy}{dt} = -\frac{my}{c_i}.$$

Integrating

$$y = e^{-mt/c_i}.$$

On plotting $\log_{10}(y/z)$, where z is the haemolymph activity at the start of the experiment, against t a straight line falling with time should be obtained (cf. Fig. 2). When y/z has fallen to e^{-1} (0·37) of its original value, $m = c_i/t$. Thus the total ion efflux (m) can be easily found.

In the above analyses the animal is treated as a one-compartment system. This is justified by the fact that the volume of the haemolymph is apparently a large fraction of the total volume of the animal (Croghan, 1958*b*).

MATERIAL AND METHODS

Adult *Artemia* as described previously (Croghan, 1958*a*) were used. The experiments were done within the temperature range 19–24° C.

The isotopes ^{24}Na and ^{82}Br were obtained from Harwell in the form of neutron-irradiated $NaHCO_3$, NH_4Br, or NaBr. Standard counting equipment was used: a G.E.C. G.M.4 tube mounted in a lead castle, and Dynatron power pack, probe unit and scaler.

For the influx experiments groups of animals in 150–200 ml. of medium were used. At zero time about 50 mg. of an active salt were added. The amount added was insignificant compared with the amount of salts already present in the medium, and thus would not have affected the steady state. After various time intervals animals were pipetted from the medium, rinsed quickly in distilled water and dried with filter-paper, and the haemolymph was collected as described in Croghan (1958*b*). A haemolymph sample was measured out in a micropipette (vol. *c.* 1 μl.), transferred to a drop of water on a small planchet and dried under an electric fire. The count rate was then determined. A single animal gave enough haemolymph for a determination. A sample of the medium was also counted.

In the efflux experiments animals were 'loaded' by soaking them overnight in an active medium similar to the media used for the influx experiments, and then the rate at which activity was lost from the animals when they were washed in an inactive, but otherwise closely similar, medium was determined. In some experiments the loaded animals were transferred to a large volume of inactive medium and after various time intervals haemolymph samples were obtained and counted as in the uptake experiments. In most of the efflux experiments using ^{24}Na a simpler procedure was used. About twenty animals were pipetted into a thin-walled Pyrex tube (9 mm. diam.). The Pyrex tube had rubber bungs at both ends through which passed short lengths of 2 mm. diam. glass tubing. To the outer ends of these were attached fine rubber tubes that led out to a reservoir and to a waste bottle. The outflow tube had a bolting silk cap to prevent animals being carried through. The

tube was clamped in a V-saddle close under the window of the G.M. tube, and inactive medium was continuously passed through (*c.* 5 ml./min.). All the difficulties of taking haemolymph samples at intervals and counting them individually are avoided. One is working with the same large group of animals continuously and can measure their total activity at close time intervals. The procedure is not only more convenient but, judging by the close linearity of the results, more accurate. It has the further advantage that the nature of the washing medium can be easily changed if desired during the experiment.

Most of the experiments were done with ^{24}Na and a few with ^{82}Br or with both isotopes together. The irradiated bromide contained also the short-lived isotope ^{80}Br, but by the time the actual counting was carried out the activity of this had become negligible. When both ^{24}Na and ^{82}Br were present the sample was counted directly and then recounted immediately afterwards with a brass filter (110 mg./cm.^{2}) between the sample and the G.M. tube. The filter was calibrated separately with ^{24}Na and ^{82}Br samples and gave good separation. It was simple to set up two simultaneous equations and to solve them to find the separate activity of both ^{24}Na and ^{82}Br in a mixed sample.

All results were corrected for resolving time, background and decay. Sufficient counts were obtained to keep the statistical error well under $\pm 3\%$.

Chemical analyses were also made on media and haemolymph samples using the methods previously described (Croghan, 1958*b*).

RESULTS

Sodium flux

The extent of exchange of haemolymph sodium was studied. Some animals were placed in a NaCl solution containing tracer amounts of ^{24}Na and left for 12 hr. Three samples of haemolymph (each obtained from six animals) and a sample of the medium were taken, and the activity and sodium concentration determined in each. Each sample was analysed in duplicate or triplicate. The results are summarized in Table 1. The specific activity (counts per minute (c.p.m.)/mM. Na) of the haemolymph had risen and reached a value which, within the limits of accuracy, is the same as that of the medium. Thus the haemolymph sodium is rapidly and completely exchangeable with that of the medium.

TABLE 1. *Exchange of haemolymph sodium*

Material	c.p.m./unit volume	mM. Na/l.	c.p.m./mM. Na
NaCl solution	4272	592	7·22
Haemolymph samples	1198	164	7·30
	1180	160	7·37
	1162	162	7·20

The rate of uptake of ^{24}Na has also been studied in animals adapted to media ranging from 10 to 600% sea water. In these media the animals were in a steady

state. A little $^{24}NaHCO_3$ was added to the medium, and after various time intervals samples of haemolymph were taken from individual animals and their activity determined. A typical uptake curve is plotted in Fig. 1, which is for animals in sea water. The fluxes were calculated and expressed as mM. Na/l. of haemolymph/hr.

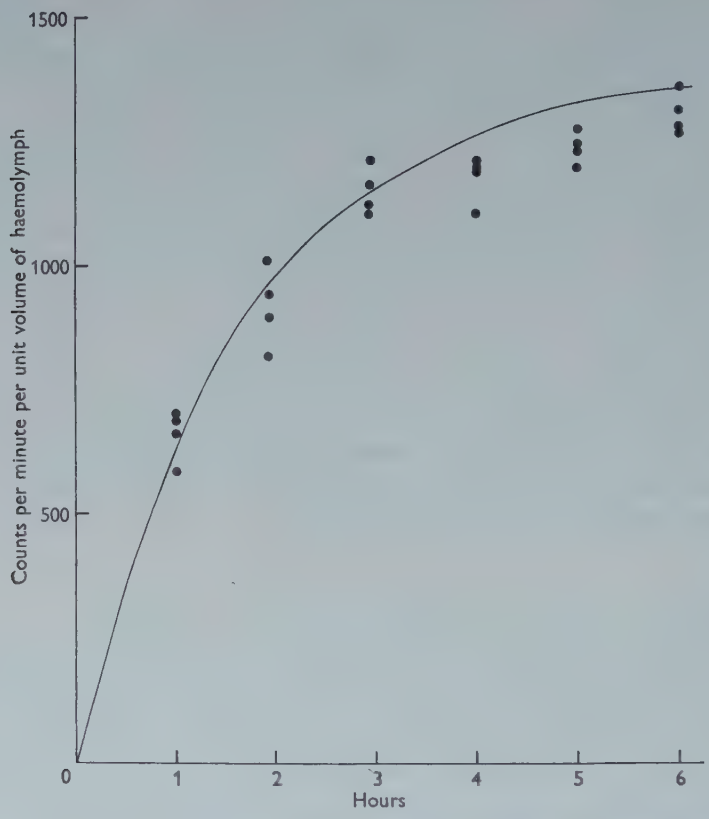

Fig. 1. Uptake of ^{24}Na from sea water. Each point represents a single animal.

The accuracy of the values from the more concentrated media tends to fall since the sodium concentration in the haemolymph is very much less than in the medium and thus the counting rate of the haemolymph samples is low. In addition, the influx appears much greater in the more concentrated media, making it more difficult to obtain accurate values.

The rate of loss of ^{24}Na has also been studied in animals adapted to media of various concentrations. In most of these experiments the total activity remaining in a group of animals while inactive medium was flowing past was determined at intervals. An example of a washing-out curve is given in Fig. 2, which is for animals being washed with sea water. On the assumption that all the body sodium is in the haemolymph, the fluxes were calculated and expressed as mM. Na/l. haemolymph/hr.

As both the influx and efflux experiments were carried out under steady-state conditions, the results should be the same. All the results are plotted in Fig. 3.

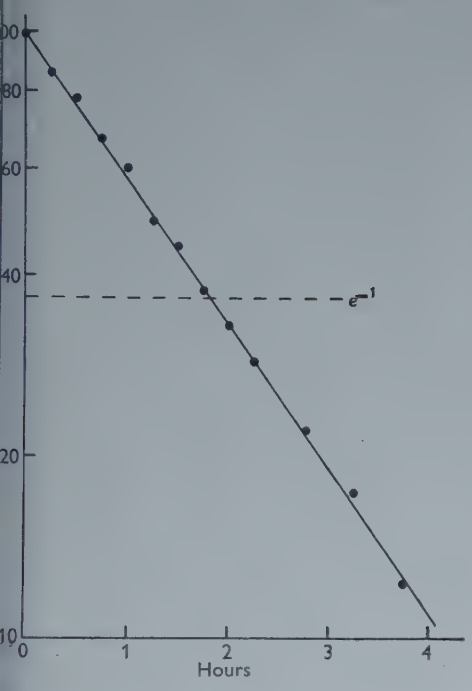

Fig. 2

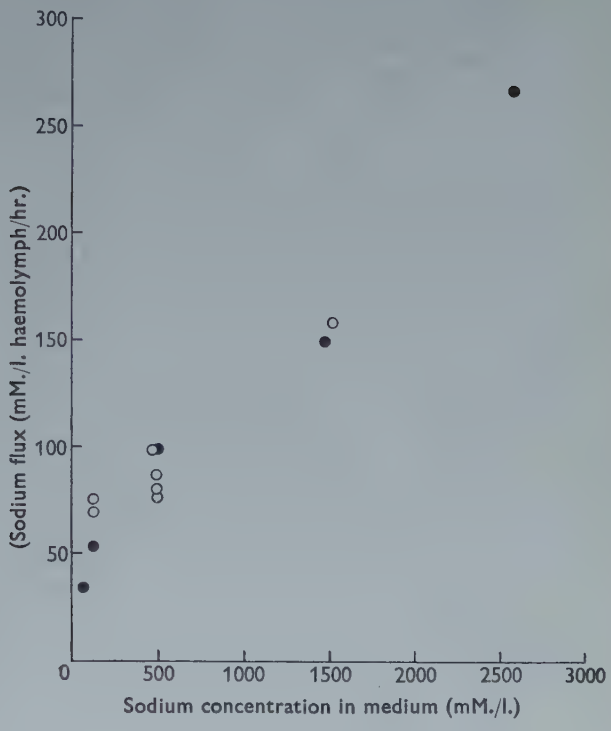

Fig. 3

Fig. 2. Loss of ^{24}Na from a group of animals washed with inactive sea water.

Fig. 3. Relation between the total sodium flux and the sodium concentration in the medium. Closed circles are influx experiments and open circles are efflux experiments.

Chloride flux

An attempt has been made to measure chloride flux using ^{82}Br as a label. The extent of exchange of bromide with chloride was studied. Some animals were transferred to sea water containing a small amount of $NH_4{}^{82}Br$. After 27 hr. two samples of haemolymph (each obtained from nine animals) and a sample of the medium were taken, and the activity and halide concentration determined in each. All analyses were done in duplicate. The results are summarized in Table 2. The specific activity (c.p.m./mM. halide) of the haemolymph had risen rapidly to a value which, within the limits of accuracy, is the same as that of the medium. In a further experiment animals were transferred from sea water to a NaBr solution containing some ^{82}Br. No chloride was present. After 27 hr. a haemolymph sample (obtained from seven animals) and a sample of the medium were taken and analysed as before. The results are also summarized in Table 2. They indicate that the haemolymph chloride is rapidly and completely exchanged with, and replaced by, bromide. These animals were still quite active.

Since the ratio Br/Cl in the haemolymph rapidly becomes the same as in the outside medium it is reasonable to consider that ^{82}Br is a satisfactory label for

TABLE 2. *Exchange of haemolymph chloride with bromide*

Material	c.p.m./unit volume	mM. halide/l.	c.p.m./mM. halide
Sea water	8430	585	14·40
Haemolymph samples	2170	147	14·75
	2260	152	14·85
NaBr solution	5610	514	10·90
Haemolymph sample	1254	117·5	10·70

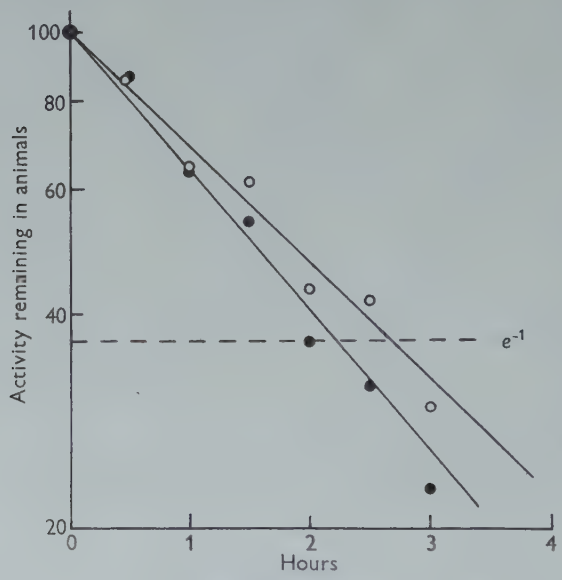

Fig. 4. Loss of ^{24}Na and ^{82}Br from animals washed in inactive NaCl solution. Closed circles refer to ^{24}Na and open circles to ^{82}Br.

chloride, and the results already quoted show that the haemolymph chloride exchanges rapidly and completely with that of the medium.

As the significance of a comparison of the fluxes of halide and sodium is much greater if they can be determined at the same time on the same group of animals, both ^{82}Br and ^{24}Na were used together. 30 mg. each of $^{24}Na^{82}Br$ and $^{24}NaHCO_3$ were added to 100 ml. of sea water. Animals were left in this medium overnight. The animals were then transferred to an inactive 500 mM./l. NaCl solution (closely isotonic with sea water). At intervals samples of the haemolymph (each obtained from five animals) were taken. The separate activities of ^{82}Br and ^{24}Na were determined in each sample. The residues of the haemolymph samples were then all pooled and the sodium and chloride concentrations were determined. The fall of haemolymph activity is plotted in Fig. 4, and the results are summarized in Table 3. The fluxes of both ions are very fast and of the same order, but the chloride flux is lower than that of sodium. It would be unwise to claim too much significance

for this difference, as there is always the possibility that the rate of bromide exchange is not quite the same as that of chloride.

TABLE 3. *Efflux of sodium and bromide from the haemolymph*

Ion	Time constant (hr.)	Haemolymph concentration (mM./l.)	Total efflux (mM./l. haemolymph/hr.)
Na	2·25	172	76·5
Cl	2·68	153	57

Ion influx in ligatured animals

Some animals from a sea-water medium were prevented from swallowing by being ligatured at the neck and anus with fine strands teased out of bolting silk. They were kept overnight in sea water. Then both unligatured and ligatured animals were transferred to a 516 mM./l. NaCl solution containing a little ^{24}Na and ^{82}Br. Haemolymph samples from individual animals were counted to obtain the separate activities of ^{24}Na and ^{82}Br in the haemolymph. The haemolymph residues were then pooled to form two samples: one from unligatured animals and the other from ligatured animals, and the sodium and chloride concentrations were determined. The ^{24}Na and ^{82}Br activities were also determined on a sample of the medium. There was no significant difference of sodium and chloride concentrations in the haemolymph as between the ligatured and unligatured animals. To make the ^{24}Na and ^{82}Br results comparable the relative activity of the haemolymph, $c_o y/x$, where y is the activity in the haemolymph, x the activity in the medium and c_o is the concentration of NaCl in the medium, is plotted in Fig. 5. There appears to be no significant difference between the two groups of animals.

Sodium efflux in sodium-free media

Animals were loaded by soaking in sea water containing ^{24}Na and the total activity remaining in a group of animals as inactive medium was flowing past was determined at intervals. In the experiment recorded on Fig. 6, the active animals were first washed with inactive sea water. Then the washing medium was changed to an erythritol solution (12·8%, closely isotonic with sea water). Later the washing medium was changed back to sea water. In the erythritol solution there was a very sharp sudden decrease in the efflux. The efflux increased sharply again when the medium was changed back to sea water. A similar sharp decrease in efflux has also been seen in distilled water. In similar experiments (Fig. 7) animals from a 25% sea-water medium (closely isotonic with the haemolymph) were loaded with ^{24}Na and then washed with inactive 25% sea water. On changing the washing medium to distilled water, there was again the sudden decrease in efflux.

Exchange of potassium

As high concentrations of potassium are rapidly toxic to *Artemia*, and as there appears to be a competitive effect between potassium and sodium ions (Croghan, 1958*a*), it was considered of interest to study the potassium exchange. Animals

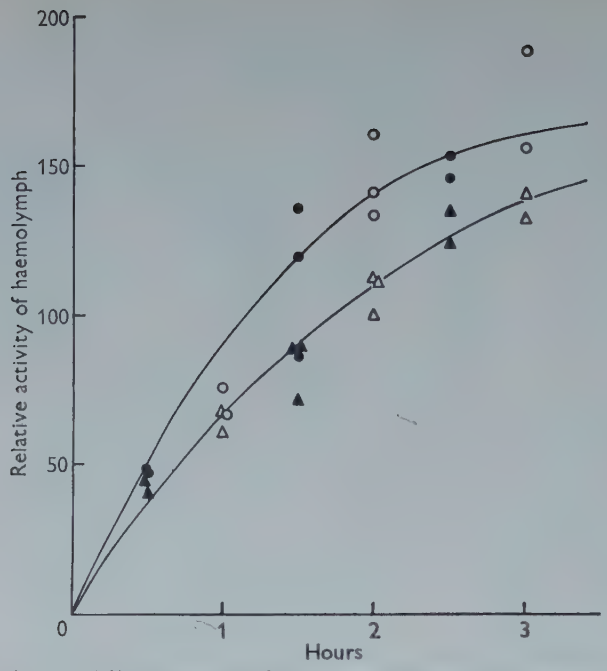

Fig. 5. Uptake of ^{24}Na and ^{82}Br from a NaCl solution. Uptake of ^{24}Na by unligatured animals, ●; uptake of ^{24}Na by ligatured animals, ○; uptake of ^{82}Br by unligatured animals, ▲; uptake of ^{82}Br by ligatured animals, △.

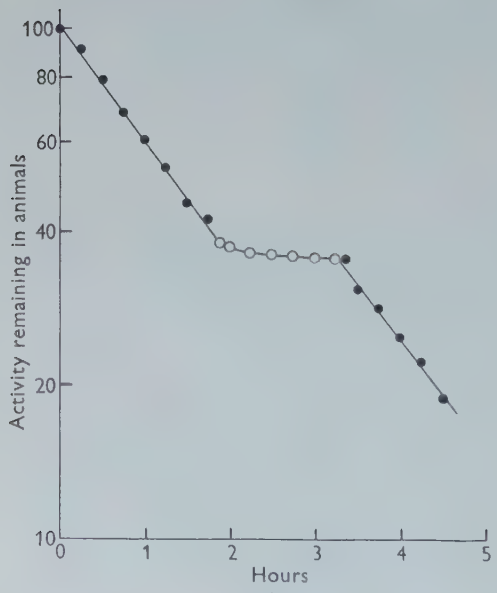

Fig. 6

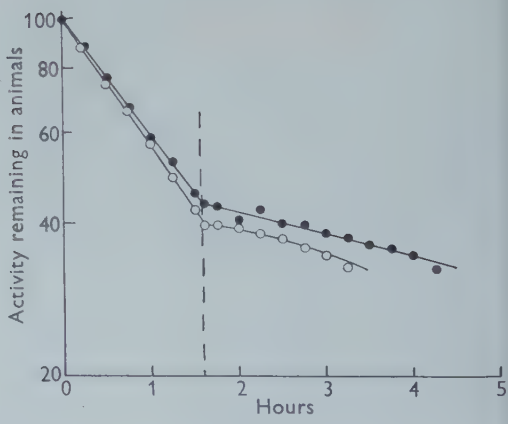

Fig. 7

Fig. 6. Loss of ^{24}Na from a group of animals washed with inactive media. Washing medium was sea water during time represented by closed circles and erythritol solution during time represented by open circles.

Fig. 7. Loss of ^{24}Na from two groups of animals (closed and open circles) washed with inactive media. Washing medium 25 % sea water until time represented by vertical broken line, when washing medium was changed to distilled water.

were taken from a sea-water medium and transferred to a 550 mM./l. solution of potassium benzenesulphonate. Haemolymph samples (each obtained from six to eight animals) were taken at the start and at 30 min. intervals, and the osmotic pressure, sodium, potassium and chloride concentrations were determined. By 30 min. all the animals were very moribund. The results are summarized in Fig. 8. All that is happening is a fairly rapid 1:1 exchange of potassium for sodium. The rapid rise in haemolymph potassium concentration easily explains the rapid toxicity of potassium rich media.

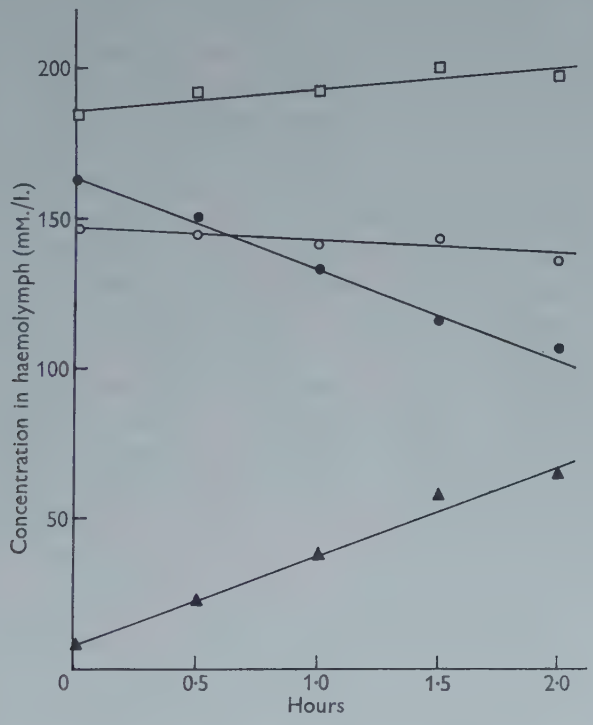

Fig. 8. Changes in haemolymph composition in a solution of potassium benzenesulphonate. Osmotic pressure, □; sodium concentration, ●; chloride concentration, ○; potassium concentration, ▲.

DISCUSSION

The first point of interest in the results is the extremely fast flux that has been found. We may compare these results from *Artemia* (weight about 7–10 mg. each) with those that have been found by tracer methods in other animals of comparable size. Holm-Jensen (1948) for *Daphnia magna* found an influx of 11 mM. Na/l. haemolymph/hr. (5·5 mM. Na/kg. animal/hr.), and for the little teleost *Lebistes reticulatus* an influx of 1·4 mM. Na/kg. animal/hr. From the data of Treherne (1954) it is possible to calculate that for *Aedes aegypti* the influx is about 1·1 mM. Na/l. haemolymph/hr. These other species are all freshwater forms. The flux in *Artemia* is enormously faster than in these other species.

It is of interest to consider the significance of the high ionic flux in *Artemia*. The sodium flux increases rapidly as the external concentration is raised and this indicates that the fast flux is dependent on a high concentration of this ion in the medium. The simplest explanation of these steady-state exchanges is that they represent diffusion movements and balancing active mechanisms. In a permeable animal, in a medium of a different concentration from the haemolymph, the ratio of the passive diffusion fluxes in both directions is: $m_i/m_o = c_o/c_i$, where m is the passive flux and c the concentration. The difference between the two passive fluxes $(m_i - m_o)$ is the net amount of ion that enters per unit time and has to be actively transported against the concentration gradient in order to maintain the steady state. It can be seen that the flux should increase proportionally to the increase of the concentration in the medium. The net influx $(m_i - m_o)$ should also increase similarly. The minimum thermodynamic energy required to excrete actively an amount equal to this net influx is $RT \log_e (c_o/c_i)$ cal./M. moved from c_i to c_o. With the maximum rate of exchange found in *Artemia* ($m = 268$ mM. Na/l. haemolymph/hr.; $c_i = 274$ mM. Na/l.; $c_o = 2570$ mM. Na/l.) this work would equal 320 cal./l. haemolymph/hr. In more dilute media this work would be much less, and would be zero when $c_i = c_o$ whatever the actual flux. These results can be compared with the total energy available from respiratory metabolism. Kuenen (1939), Eliassen (1952) and Gilchrist (1954) have all given data on oxygen uptake in *Artemia*. Using a value of 0·69 ml. oxygen/g. animal/hr. from Gilchrist (1954), and assuming that all the measured flux represents diffusion and balancing active transport, it can be calculated that, even with the highest rate of exchange observed, the minimum thermodynamic energy for NaCl excretion is only about 6% of the total available metabolic energy. Active transport of this magnitude could thus be well within the metabolic capabilities of *Artemia*.

The more detailed studies, however, have raised objections to the obvious and straightforward interpretation outlined above. The ligaturing experiments showed that there was little difference in the influx of ^{24}Na and ^{82}Br as between ligatured and unligatured animals. This strongly suggests that most of the rapid ionic fluxes are occurring across the outer surface of the body (branchiae?), and not appreciably across the gut epithelium. This is curious as it has been claimed (Croghan, 1958*d*) that most of the net entry of NaCl into the haemolymph takes place across the gut epithelium. This suggests that the flux across the external surface is so fast that it masks the contribution of the gut, and also raises doubts as to whether this flux across the external surface is really a simple diffusion process.

Further doubts arise when we consider the effect of changing the washing medium during efflux experiments. When the medium is changed from sea water or 25% sea water to a sodium-free medium the sodium efflux immediately drops to a very low value. This indicates that for sodium efflux to occur sodium ions must be present outside. It suggests that under steady-state conditions the influx and efflux of sodium are not independent processes but are a closely coupled 1:1 exchange between the medium and haemolymph, i.e. some type of exchange diffusion system. It appears that potassium ions in the medium can to some extent substitute for

sodium in this exchange process. The competitive effect between potassium and sodium in the medium (Croghan, 1958*a*) also supports this view.

Ussing (1947) and Levi & Ussing (1948) used the term 'exchange diffusion' for a system in which a fast exchange of an ion between two compartments in which it was not in electrochemical equilibrium could occur as a purely passive process (i.e. no work is done and no energy is required). They visualized a membrane impermeable to the ion concerned and containing scattered particles of an ion-exchange material with a high affinity for this ion. These particles move thermally between the inside and outside of the impermeable membrane. Ions attached to the exchange particles could exchange with the ions present in the solutions on either side of the membrane. The fluxes of this ion in both directions are not independent processes and a 1:1 exchange of this ion between the compartments on either side of the membrane should occur. It is not necessary to presuppose the existence of any special carrier for the above process, and any charged component of a cell membrane capable of thermal oscillation or rotation could presumably have this property.

Consider also a system in which, although the concentration of an ion is different, the electrochemical potential of an ion is the same on both sides of a membrane permeable to this ion alone (i.e. a Donnan equilibrium). If one of these ions moves thermally across the membrane, another similar ion must move across in the opposite direction, otherwise the system would move away from equilibrium. The fluxes of these ions in both directions are not independent and a 1:1 exchange of this ion would occur between two compartments in which the concentration of this ion was different. Such a situation would occur, for example, with the exchange of potassium between cell fluids and the cell medium. A model having such properties can be set up by separating two different concentrations of an electrolyte by a membrane of an ion-exchange material (i.e. either selectively anion or cation permeable). Such a model is likely to have biological analogues, as fixed charges are likely to be quite common in biological membranes.

Whatever the details of the actual exchange diffusion process concerned, it would be expected from Mass Law considerations that the flux would be proportional to the concentrations of the exchanging ion on either side of the membrane. The flux would increase with the concentration of the external medium, and when this medium is replaced by one in which the exchanging ion is absent the flux into this medium would immediately fall to a low value.

It appears probable that the main part of the fast flux in *Artemia* is due to exchange diffusion, although it is difficult if not impossible to obtain absolutely unambiguous evidence in such a small closed system. A net diffusion and active ion transport must also be occurring, but, if this is relatively slow compared to exchange diffusion, its contribution to the total rate of ion exchange would be masked. The exchange of chloride in *Artemia* appears to be nearly as fast as that of sodium. It is probable that both these ions are undergoing exchange diffusion. The simplest picture is to imagine a membrane with a mosaic of separated exchange diffusion sites.

Exchange diffusion itself can have no significance or value to the living system,

for as all ions of a given type are identical, and as no energy is required, the system can have no awareness that these ions are rapidly exchanging. For this reason no selective influences will operate on exchange diffusion as such. Exchange diffusion may be regarded as a harmless by-product of the properties of cell membrane constituents. The possibility of exchange diffusion, however, complicates the interpretation of tracer studies, especially in such systems as small intact animals, and great care is needed in interpretation.

SUMMARY

1. The ionic fluxes between the medium and haemolymph have been studied in adult *Artemia salina*, under steady-state conditions using ^{24}Na and ^{82}Br.

2. Extremely high fluxes have been found, the flux increasing markedly in the more concentrated media.

3. The results are interpreted as indicating that both the sodium and chloride in the haemolymph are undergoing rapid exchange diffusion with the sodium and chloride in the medium.

I wish to thank Dr J. A. Ramsay, F.R.S., for his interest and advice. I also wish to thank the Department of Scientific and Industrial Research for a maintenance grant.

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ACOUSTIC REFLEXION EXPERIMENTS WITH PERCH (*PERCA FLUVIATILIS* LINN.) TO DETERMINE THE PROPORTION OF THE ECHO RETURNED BY THE SWIMBLADDER

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INTRODUCTION

Cushing & Richardson (1955) found that the echo returned by artificial swimbladders hung alongside dead cod suggested that from 35 to 70% of the echo energy returned by a normal fish would come from the swimbladder. However, they were not able to measure the amount of energy reflected from a single fish with and without its swimbladder. Laboratory experiments have been carried out to look further into this matter, their immediate purpose being an attempt to determine, under controlled conditions, the contribution of the swimbladder to the strength of an echo returned by a fish.

METHOD

The experiments were carried out at Kelvin and Hughes Ltd., Barkingside, Essex, in an indoor freshwater tank of dimensions 6 m. long, 3 m. wide and 3 m. deep. The amplitude of the echo returned by a freshly killed fish suspended in the tank was measured before and after the removal of the gas from the swimbladder. The effect of substituting a false swimbladder after the removal of the real one was also examined. The transmitting and receiving transducers and the target system were placed on the long axis of the tank. The transducer system was 1·5 m. from one end of the tank and the distance between the transducers and the fish 3·3 m.

(i) *The choice of the fish for a target.* The experiments could only be carried out on dead fish of a size consistent with the physical limitations of the whole system, which are discussed later. It was necessary to select a freshwater fish about 20 to 30 cm. in length which would not lose any appreciable amount of gas from the swimbladder for 2–3 hr. after death. The perch, *Perca fluviatilis*, seemed the best choice, since it is hardy, is easily kept in captivity, and has a well-developed closed swimbladder occupying about 7% of its total volume (Plattner, 1941; Jones, 1951) which does not lose any significant amount of gas during the first few hours after death.

Twenty perch, whose lengths ranged from 16·5 to 24·0 cm., netted in 2 to 3 m.

of water, were obtained from dealers and kept in excellent condition in tanks until required.

(ii) *Electro-acoustic apparatus.* The electro-acoustic equipment consisted of a valve transmitter from a standard Kelvin-Hughes MS. 28 echo sounder, two MS. 28 transducers acting as the projector and hydrophone units, and a Cossor Model 1049 Mk. III oscilloscope.

The valve transmitter gave an electrical output of 400 W. in pulses, the lengths of which could be chosen to be 0·5, 1·0 or 2·5 msec. as required. The pulse repetition rate was 200 per minute.

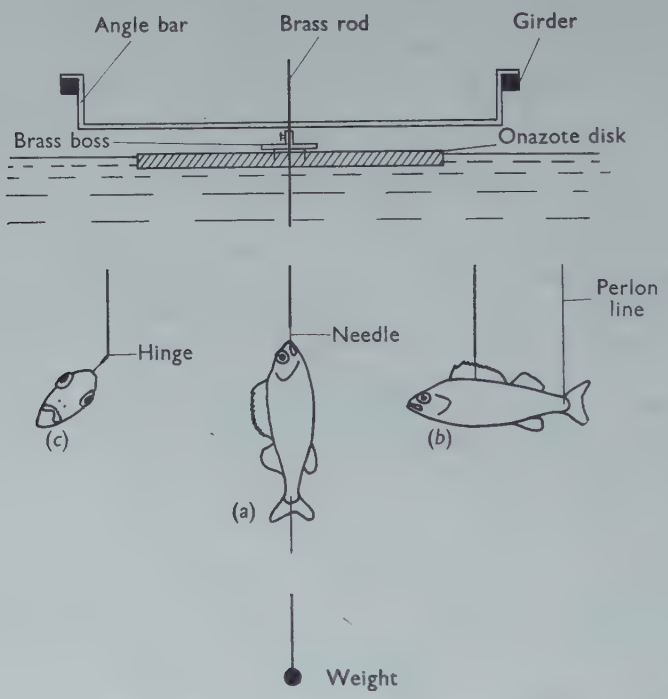

Fig. 1. Diagram to show the method used to suspend the fish in the tank.

The MS. 28 transducers acting as projector and hydrophone were operated at their resonant frequency of 30 kc./s. The radiating face of each was of dimensions 14·5 × 9 cm. (approximately $3\lambda \times 2\lambda$ at 30 kc./s.). These units were mounted one above the other with their longer sides adjacent, so that the centre of this projector-hydrophone system was 1·5 m. below the water surface. This system was fixed rigidly relative to the sides of the tank. The projector was connected to the MS. 28 echo sounder valve transmitter; such a system produced an acoustic output of 160 W. The hydrophone was connected to the amplifier input of the oscilloscope, and the received signals were displayed on the cathode-ray tube in A-scope presentation.

(iii) *Target suspension.* The target was held in mid-water on a straight brass rod 0·16 cm. in diameter as shown in Fig. 1. The rod was secured by a grub screw to

a brass boss locked by spikes to a disk of 60 cm. diameter floating on the water surface. This disk was made of 'Onazote', a rigid foamed ebonite material in which the individual air cells are isolated from one another. The free end of the rod ran through a slot in an angle bar clamped to girders running across the top of the tank. This arrangement allowed the target to be kept at a constant depth, and also allowed the vertical axis of the target suspension system to be fixed rigidly with respect to the projector-hydrophone system. The floating disk, boss and rod could be turned to rotate the target relative to the transducers, the angle of rotation being read from a degree scale marked on the 'Onazote' disk.

The target fish was impaled on the point of a darning needle soldered to the end of the brass rod. The needle was run into the skull or dorsal flesh of the fish as shown in Fig. 1 according to whether rotation was to be carried out about the longitudinal or vertical axis of the fish. The needle was held firmly into the fish by a loop of 'Perlon' of diameter 0·05 cm. through the body. This thread was then passed through the eye of the needle and was drawn tight. This method of suspension proved very successful and the fish followed the rotation of the 'Onazote' disk well. In the experiments involving rotation about its longitudinal axis the fish was kept vertical in the water by means of a small weight attached to the tail as shown in Fig. 1*a*. During rotation about the vertical axis of the fish it was necessary to support the tail after emptying the swimbladder. This was done by a 'Perlon' line (Fig. 1*b*) running up to a slit cut in the 'Onazote' disk. In some experiments involving rotation about the vertical axis the fish was fitted at an angle to the vertical to see what effect this would have on the strength and pattern of the returned signals. This was done by making a small hinge in the brass rod immediately before the point at which the darning needle was soldered to it (Fig. 1*c*). The hinge was held at any required angle of tilt by a low melting-point solder, adjustment being made with an electric soldering iron with the hinge clear of the water but the fish below the surface.

(iv) *False swimbladders.* The false swimbladders used throughout the experiment were made of 'Onazote'. This has an acoustic reflexion coefficient at 30 kc./s. of the order of 0·95. In the first experiments blocks of 'Onazote' were modelled into the shape of a cigar similar to that of the swim bladder, but in the later ones it was found more satisfactory to cut cylindrical cores from blocks with a cork borer of suitable diameter and then to cut these down to the required length.

(v) *Experimental procedure.* The fish were killed as required by prolonged immersion in 10% urethane. After an hour the fish, which was now floating in the water, was removed and quickly attached to the supporting rod and 'Perlon' lines. This took 3–4 min. and the fish was kept moist while this was done. The fish was then lowered into the water, care being taken to see that no air bubbles remained trapped within the mouth or under the gill covers. The rod and attached brass boss were locked on to the 'Onazote' disk and the rod engaged into the slot in the clamped angle bar. A series of readings was then taken.

The fish was lined up so that a tail-on or dorsal surface-on position conformed to the zero of the degree scale marked upon the 'Onazote' disk. For this position,

the amplitude of the received signals as displayed upon the oscilloscope was measured. The target system was then slowly rotated through a known angle and when the system was stationary the amplitude of the received signal was again measured. This was repeated at equal angular intervals for a 360° rotation of the fish. During rotation about the longitudinal axis measurements of the returned signal were made every 10° during anticlockwise rotation. In the experiments involving rotation about the vertical axis measurements were made every 3° during anticlockwise rotation.

After a complete rotation of 360° the rod, boss, disk and fish were disengaged from the angle bar and brought to the side of the tank and the fish was raised to a few centimetres below the surface. This was done as a check and control measure against any variations that might occur due to disturbance of the positioning of the fish when it was brought to the surface to empty the swimbladder. The system was then returned to its previous position and a second complete set of measurements made. The fish was again brought to the side of the tank and the left side cut through with the fish under water to release the gas from within the swimbladder. Every precaution was taken to ensure that no gas remained within the body of the fish. Another set of measurements was made, the target system was again moved to the side of the tank and up to the water surface, back to the centre of the tank and the observations were repeated. Next, a false swimbladder made of 'Onazote' and of approximately the same dimensions as the normal one was slipped into the body of the fish and two further sets of measurements made. Finally one series was made with the false swimbladder alone. A complete experiment thus gave duplicates of the polar diagrams for the fish with the swimbladder full, with the swimbladder empty, the fish with a false swimbladder and one of the false swimbladder alone. Experiments were done with nine fish rotated about the longitudinal axis and five fish rotated about the vertical axis. All these experiments were done using a pulse length of 0·5 msec. In each main set of experiments one was repeated with a pulse length of 1·0 msec.

(vi) *Precautions and limitations of the measurements.* The possible sources of error due to the acoustic limitations of the system can be of importance, and care was therefore taken to reduce these errors to a minimum.

(*a*) The target must be uniformly irradiated by the sound energy. Precautions were therefore taken to ensure that the measurements were carried out within the Fraunhofer region of the transducer sound field, and that the fish occupied part of this region where the sound field was uniform. Measurement showed that the fish was always within a sphere of diameter 30 cm. within which the sound intensity level did not vary by more than 1·0 db.

(*b*) The chief disadvantage of working in what is effectively such a small tank is the reverberation produced due to successive reflexions within the tank. If the target echoes are well above the reverberation level, then the interference between the target echoes and reverberations will not affect the magnitude of the target echoes to any significant extent. If, however, the two signals are of the same order of magnitude, then the wave interference can obviously produce anomalous results.

In these experiments, the amplitudes of largest signals were 20–23 db., i.e. about sixteen times, above the reverberation level. The smallest signals were, however, of the same order of magnitude. This effect was reduced to a large extent by arranging the target system in position so that its echo occurred in a 'hole' in the reverberation pattern. The existence of such a 'hole' was observed on the oscilloscope, since the energy received by the hydrophone, for any one transmitted pulse, obeys a certain intensity-time law. Consequently, the oscilloscope trace displaying the received signals does not change from one transmission to the next. At the particular time corresponding to the 'hole' the reverberation level was 10 db. (i.e. one-third of the amplitude) below the smallest signal to be measured. This 'hole' corresponded to no signal at the transducer face, rather than to destruction interference of two or more larger signals, since moving the transducer system backwards and forwards a few wave-lengths did not produce any change in the signal level within the 'hole', and produced the correct movement of the 'hole' with respect to time.

(*c*) Care must be taken to ensure that no unwanted air is contained anywhere upon the target system. No air bubbles were allowed to remain within the mouth of the fish, or to become entrapped after the swimbladder was split and emptied, and the suspension system was wiped with detergent before immersion to remove air bubbles. The fish was only removed from the water when it was attached to the brass rod. It seemed unlikely that such a short exposure to air would dry the skin of the fish to such an extent as to trap a surface film of air on its return to water and prevent it from being completely rewetted.

(*d*) The shortest pulse available, 0·5 msec., was sufficiently long for the fish to be completely enveloped within it. Consequently, the echo received was characteristic of the fish as a whole, and could not be analysed into echoes from individual portions of the target. In any case, with the transducers used it is not possible to produce at 30 kc./s. a pulse sufficiently short to allow such an analysis to be made.

At intervals throughout the experiment the output from the projector was monitored. It was found to be constant to within $\pm 0{\cdot}8$ db. over a long period and to within much less for short periods. The over-all precision of any one series of measurements is estimated from a consideration of the possible errors to be $\pm 1{\cdot}0$ db.

As a check upon the accuracy of the measurements, an experiment was performed using as a target a hollow thin-walled sphere of diameter 20 cm. The target strength of this was measured and was compared to the calculated target strength, both these values being the strengths relative to a sphere of 2 m. radius. The measured target strength was $-24{\cdot}7$ db, and the calculated target strength was $-25{\cdot}8$ db. The discrepancy between these figures is not greater than expected.

RESULTS

(i) *Rotation about the longitudinal axis.* Two sets of measurements have been taken as representative of the whole nine sets which were made. The results corresponding to these two sets are shown in Figs. 2 and 3. The duplicate sets of readings agree

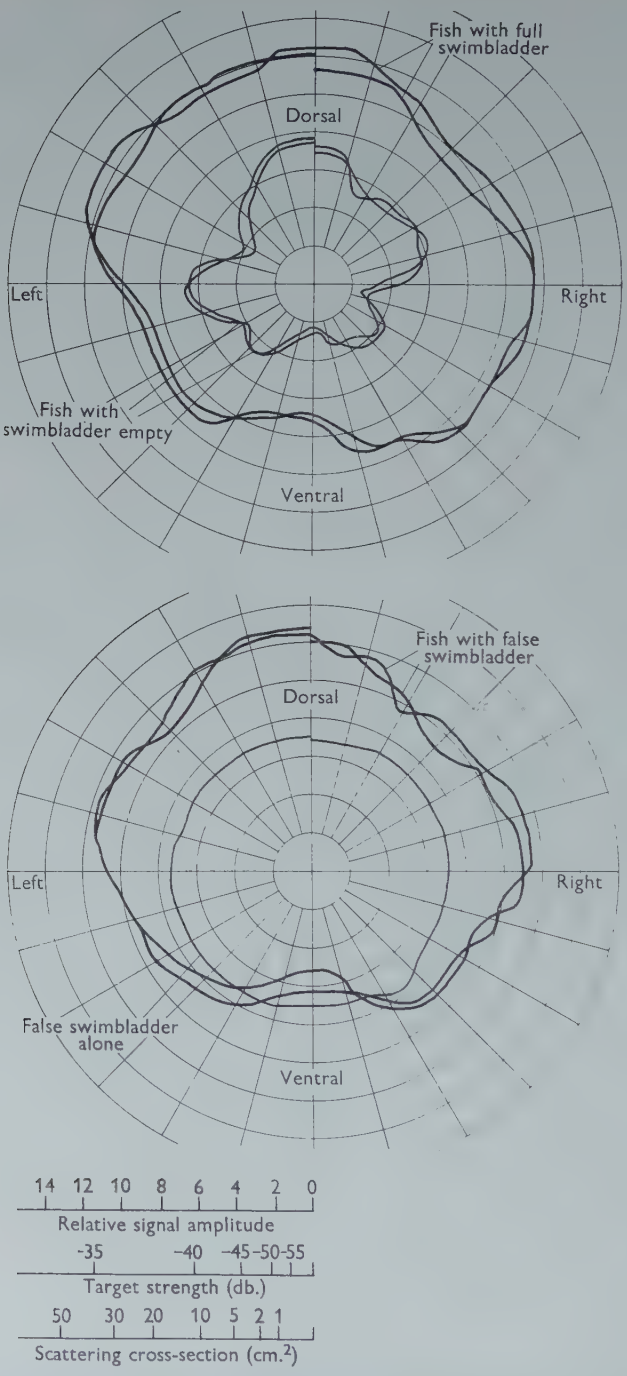

Fig. 2. Polar diagrams of relative signal amplitude, target strength and scattering cross-section obtained by rotating a fish about its longitudinal axis. Fish no. 6, 21·6 cm. long. False swimbladder 6 cm. long, 1·2 cm. diam., cylindrical, prepared with cork borer. The polar co-ordinate scales have been put below the diagrams to avoid superimposition.

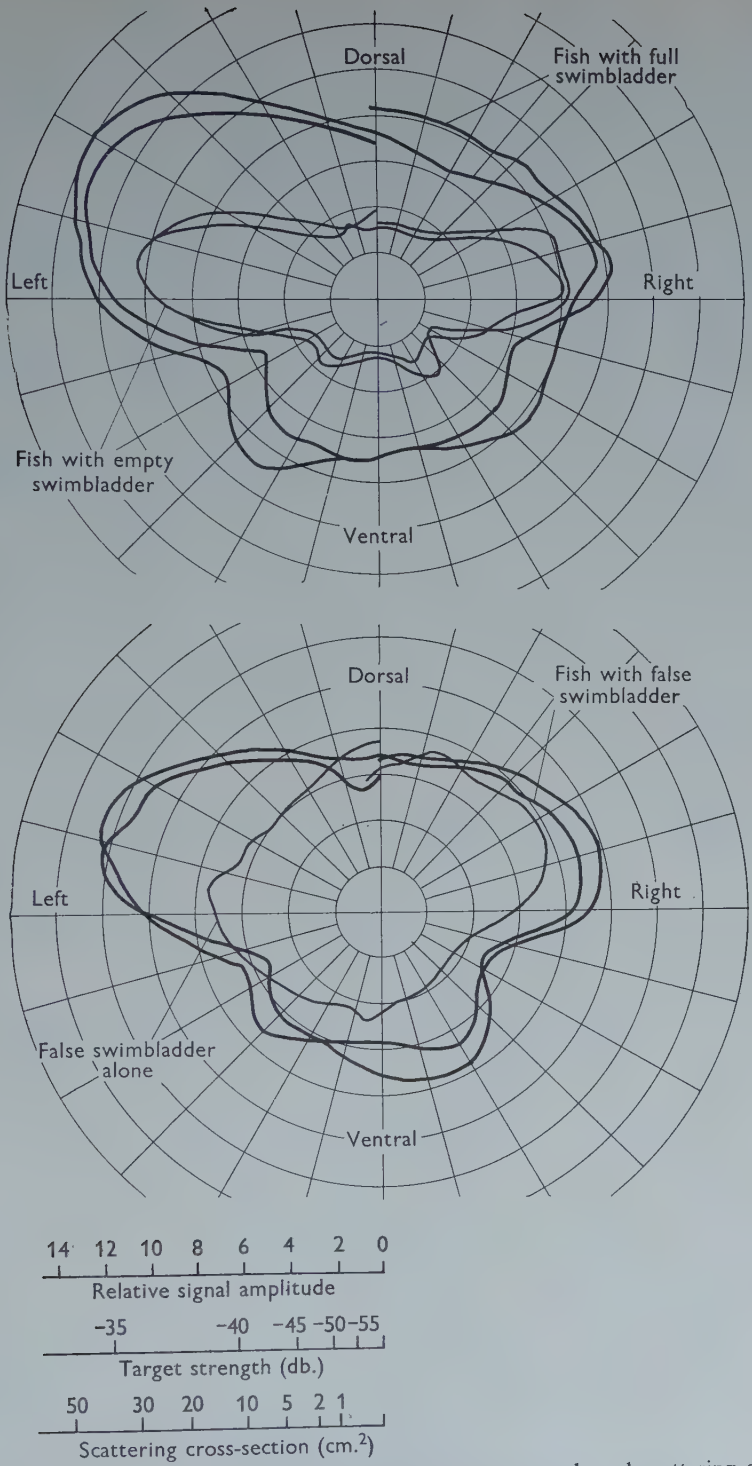

Fig. 3. Polar diagrams of relative signal amplitude, target strength and scattering cross-section obtained by rotating a fish about its longitudinal axis. Fish no. 4, 19·0 cm. long. False swimbladder 4 cm. long, max. diam. 1·2 cm., roughly cigar-shaped, cut by hand. The polar coordinate scales have been put below the diagrams to avoid superimposition.

well with one another and there is a marked reduction in the strength of the echo returned by the fish after the swimbladder is emptied. The echo signal was restored very closely to its original value by putting a piece of 'Onazote' into the space previously occupied by the swimbladder. In general, the pattern obtained by rotating the fish about the longitudinal axis approached one or other of the two shown in the figures. In Fig. 2 the signal strength is more or less uniform over the dorso-lateral region of the fish, whereas in Fig. 3 it increases as the fish comes to present its right or left side to the transducers. Variations in the form of the two polar diagrams could not be related to any difference in length or sex of the fish or to differences in the method of suspension. The difference between the polar diagrams might possibly be an effect of size. Cushing (1955) has shown with fish models that small changes in the dorso-ventral dimension can produce variations in polar diagrams. In one or two cases when the swimbladder was being punctured the fish was found to have a well-developed median ovary. After the check measurement with the false swimbladder, this ovary was removed and the measurement repeated. No measurable effect on the polar diagram of the fish was noted. The results have been summarized in Table 1 to show the reduction in echo signal amplitude following the emptying of the swimbladder when the dorsal surface of the fish is normal to the transducers and when it is 10° off to the left or right. The results show that the echo amplitude is reduced by 52% of its maximum value when the swimbladder of the fish is punctured. A change of pulse length from 0·5 to 1·0 msec. had no effect.

From the point of view of fish detection by the echo sounder the important feature of the results is the strength of the echo signal obtained from the dorsal aspect of the fish. In order to present the results in a more useful form from this point of view, each polar diagram carries three scales: (*a*) relative echo signal amplitude; (*b*) target strength in db. relative to a sphere of 2 m. radius (see Appendix); (*c*) scattering cross-section in cm.^{2} (see Appendix).

Examination of the results shows that at 30 kc./s. the target strength of the dorsal aspect for the perch, and also, therefore, for fish of similar size and structure, is about -35 to -38 db., and that the scattering cross-section of this aspect is from 15 to 40 cm.^{2}. The variations obtained, as already stated, could not be explained by physical or biological differences in the fish, nor yet by any inconsistencies in the apparatus or in the method of measurement.

(ii) *Rotation about the vertical axis.* Typical polar diagrams are shown in Fig. 4 for measurements upon: (*a*) a fish with the swimbladder full; (*b*) a fish with the swimbladder empty; (*c*) a false swimbladder alone; (*d*) a fish with a false swimbladder.

The symmetry of cases (*a*), (*c*) and (*d*), with respect to the dorso-ventral axis is noticeable, and so is their similarity in shape. From Table 2 it will be seen that the angular widths to the 1st minimum of the large lobes in cases (*a*) and (*d*) are close to those of the false swimbladder alone, case (*c*). This is a further indication that the echo from the fish used in the experiment is primarily due to the swimbladder.

Again, removal of the swimbladder has considerable effect on the strength of the signal received from the fish. One experiment was carried out to examine the effect

TABLE 1. *Relative signal strengths received from perch during rotation about the longitudinal axis*

(Pulse length 0·5 msec. unless stated.)

Fish no.	Length (cm.)	Series	Relative signal strength						Percentage reduction in amplitude
			Swimbladder full			Swimbladder empty			
			Left, 10°	Dorsal, 0°	Right, 10°	Left, 10°	Dorsal, 0°	Right, 10°	
1	22·5	(i)	84	98 90	89	45	45 48	44	47·5
		(ii)	75	90 89	96	45	48 48	50	
2	24·0	(i)	104	114 105	103	48	52 51	62	42·8
		(ii)	105	95 99	99	69	72 54	63	
3	20·5	(i)	55	64 61	60	35	27 35	37	39·6
		(ii)	50	52 48	52	29	36 30	38	
4	19·0	(i)	73	70 70	68	31	38 31	30	56·2
		(ii)	70	69 79	79	30	31 31	31	
5	21·5	(i)	145	152 150	151	46	44 53	54	64·5
		(ii)	141	145 150	152	54	52 59	59	
6	18·0	(i)	118	118 109	109	74	74 70	70	39·8
		(ii)	120	120 120	120	72	72 65	65	
7	16·5	(i)	97	97 95	95	40	40 38	38	60·2
		(ii)	99	95 95	95	38	38 37	37	
8	19·5	(i)	118	118 114	114	40	40 49	49	61·6
		(ii)	110	110 108	108	42	42 42	42	
9	20·5	(i)	125	131 131	132	59	68 65	61	52·6
		(ii)	118	128 123	125	50	60 57	60	
Pulse length 1·0 msec.		(i)	138	151 150	152	65	70 65	75	52·7
		(ii)	142	144 138	142	62	65 70	75	
							Mean reduction in amplitude		51·8

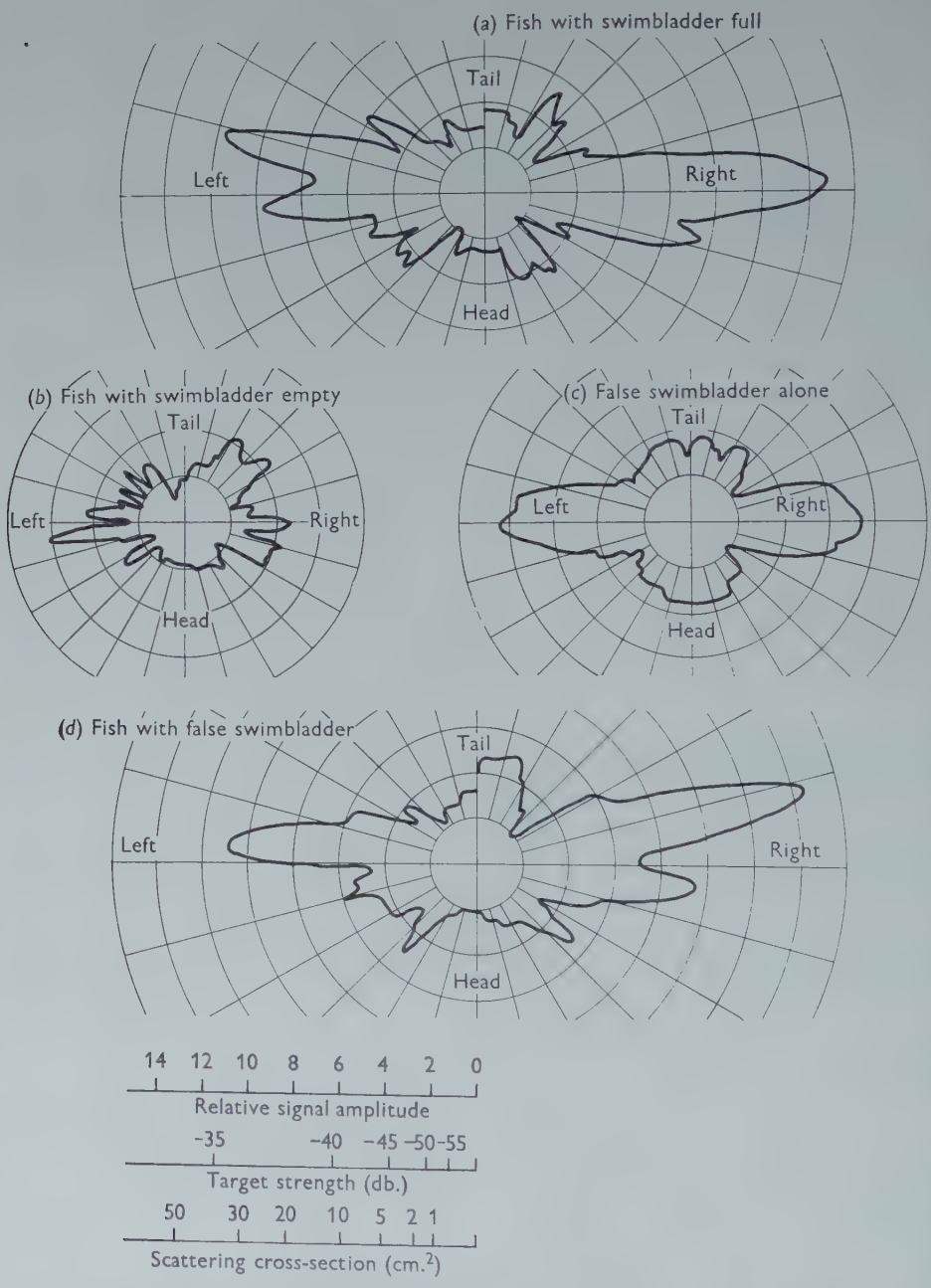

Fig. 4. Polar diagrams of relative signal amplitude, target strength and scattering cross-section obtained by rotating a fish about its vertical axis. Fish no. 3, 20·0 cm. long. False swimbladder 5 cm. long, 1·2 cm. diam., cylindrical, prepared with cork borer. Only one of the duplicate sets of results is figured in (*a*), (*b*) and (*d*). The polar co-ordinate scales have been put below the diagrams to avoid superimposition.

TABLE 2. *Comparison of polar diagrams obtained during rotation about the vertical axis*

	Fish 2. Length, 22 cm. False swimbladder: 5 cm. long, 1·2 cm. diam.		Fish 3. Length, 20 cm. False swimbladder: 5 cm. long, 1·2 cm. diam.	
	Angular width of main lobes to 1st minimum		Angular width of lobes to 1st minimum	
	Right	Left	Right	Left
Fish with natural swimbladder	54°	43°	71°	57°
Fish with false bladder	47°	52°	70°	66°
False swim-bladder alone	56°	56°	76°	67°

TABLE 3. *Relative peak signal strengths received from perch during rotation about the vertical axis*

(Pulse length 0·5 msec. unless stated.)

Fish no.	Length (cm.)	Series	Relative peak signal strengths				Percentage reduction in amplitude
			Swimbladder full		Swimbladder empty		
			Left side	Right side	Left side	Right side	
1	19·0	(i)	92 109	101 133	50 50	45 45	
		No tilt	92 109	133 124	47 42	40 40	
		(ii)	86 90	145 158	34 34	39 38	67·6
		12° tilt	89 86	151 159	34 32	38 33	
		(iii)	132 121	130 130	39 38	36 35	
		40° tilt	110 120	142 137	38 36	35 35	
2	22·0	(i)	135 135	174 169	117 109	64 64	
			132 132	169 169	92 88	59 59	
		(ii)	140 140	179 179	76 75	55 55	51·7
			132 132	158 130	69 64	61 55	
3	20·0	(i)	109 109	140 140	58 58	45 43	
			94 94	132 128	43 42	43 42	62·7
		(ii)	108 108	140 140	49 49	42 42	
			111 111	120 110	40 40	35 35	
4	21·5	(i)	152 140	134 136	102 95	99 95	
			137 127	99 74	85 73	81 75	30·0
		(ii)	140 142	134 111	104 91	78 88	
			135 119	111 94	89 81	81 65	
5	20·5	(i)	120 118	132 132	56 53	60 58	
			111 111	128 122	52 50	52 50	
		(ii)	112 113	135 125	62 60	59 59	53·3
			111 101	118 118	58 51	55 55	
	Pulse length 1·0 msec.	(i)	150 144	161 158	70 65	74 68	
			132 132	155 149	62 60	68 64	53·9
		(ii)	147 141	161 154	75 70	70 70	
			135 125	150 151	67 59	70 70	
Mean reduction in amplitude							53·2

of tilting the fish at angles of 12° and 40° to the vertical axis during rotation, but this appeared to make but slight alteration in the strength or pattern of the signals returned. The peak signals returned by the fish when beam-on to the transducer seemed a reasonable measure of the contribution of the swimbladder to the echo and the four peak signals from the left and right sides during each experiment are summarized in Table 3. It will be seen that there is a reduction of 53% in the peak signal amplitudes following the emptying of the swimbladder.

The target strength of the lateral aspect of the normal fish is about −33 db. and that of the tail-on or head-on aspect is about −45 db. The corresponding values of the scattering cross-section are approximately 60 and 5 cm.^{2}. Removal of the swimbladder reduces the target strength of the lateral aspect of the fish by about 12 db. and the scattering cross-section by a factor of 12.

DISCUSSION

The main result of these experiments is to confirm (i) that the swimbladder plays a major part in determining the acoustic reflexion properties of the fish used, and (ii) that in general Cushing & Richardson (1955) correctly estimated that of the total echo energy from a fish, about 50% is returned from the swimbladder. Both conclusions (i) and (ii) are reached by considering the echo signal amplitudes received from the fish with and without its swimbladder. In addition, conclusion (i) is strengthened by a consideration of the polar diagrams obtained for the fish with its swimbladder and comparison of these diagrams with those obtained for a swimbladder alone.

It will be noted that the measurements were made by rotating the target relative to the combined projector-hydrophone system. Consequently, the results obtained are measurements of the amount of energy reflected in the direction of its initial incidence. This is not in accord with the usual methods of measuring scattered energy. The method used here is, however, relevant to the echo-sounding detection of fish, to the study of which the results and conclusions can be applied.

SUMMARY

1. Measurements were made under controlled conditions to determine the contribution of the swimbladder to the reflexion of acoustic energy from the perch, *Perca fluviatilis*.

2. The amplitude of the echo fell by 50% when the swimbladder was emptied; this was observed both when the dorsal surface and when the lateral surface faced the direction of the incident sound energy.

3. The polar diagram of the fish with its natural swimbladder is similar in shape to that obtained with a false 'Onazote' swimbladder.

Thanks are due to Messrs Kelvin and Hughes Ltd., Barkingside, Essex, and to Mr W. Halliday, Chief Physicist, Acoustics Division of Messrs Kelvin and Hughes Ltd., for permission to carry out the work, and also to Mr W. Thomas who helped considerably in preparing the apparatus and in making the measurements.

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APPENDIX

(i) *Target strength*

For the reflexion of sound energy, it is known that

$$I_r = \frac{k}{r^2} I_0, \tag{1}$$

where I_0 is the intensity of the energy incident upon the target, I_r is the intensity of the reflected sound at a point, distance r from the target, and k is a factor whose magnitude is determined solely by the reflecting properties of the target.

The target strength T is defined as

$$T = 10 \log_{10} k, \tag{2}$$

substituting for k

$$= 10 \log_{10} \frac{I_r}{I_0} r^2. \tag{3}$$

In the experiments described the sound intensities I_r, I_0 were measured with linearly responding apparatus, so that if (for the given receiving transducer) V_0 and V_r are the received voltages corresponding to I_0 and I_r, respectively,

$$T = 10 \log_{10} \left(\frac{V_r}{V_0} r \right)^2. \tag{4}$$

Now r and I_0 (and so V_0) were constant throughout the experiments so that

$$T = 20 \log_{10} A V_r, \tag{5}$$

where $A = r/V_0$. The target strength is essentially a measure at a certain point of the level of the energy reflected from the target compared to that of the incident energy, the dispersion of energy due to the inverse square law being allowed for. The reference level is given by a target for which $k = 1$. It can be shown that a sphere of radius equal to 2 units of dimension in the dimensional system used is such a target. In these experiments the target strengths are referred to a sphere of radius 2 m. as reference target.

(ii) *Scattering cross-section*

The scattering cross-section σ_s is defined as that area perpendicular to an incident plane beam of sound energy such that the energy flowing through it is equal to the total energy scattered by the target in all directions. Thus, using the same notations as above,

$$4\pi r^2 I_r = \sigma_s I_0, \tag{6}$$

or

$$\sigma_s = \frac{4\pi r^2 V_r^2}{V_0^2} = BV_r^2, \tag{7}$$

where B is a constant, since r, V_0 were kept constant throughout the experiment.

Further discussion of these concepts is to be found in 'The physics of sound in the sea', *Summary Technical Report of Division* 6, *NDRC*, **8**, 1946 (Washington, D.C.), parts III and IV.

THE MECHANISM OF OBJECT LOCATION IN *GYMNARCHUS NILOTICUS* AND SIMILAR FISH

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INTRODUCTION

Present observations suggest a correlation between the electrical discharges emitted by certain species of fish and their sensory perception.

The theory postulated for this mechanism of perception implies that the potential distribution over the surface of the fish is detected by a series of receptors; this information is then interpreted to indicate the position of objects with a conductivity differing from that of water (Lissmann, 1951, 1958). Although the theory is supported both by morphological and physiological evidence, the quantitative physical aspects appear to involve an unusually high degree of electrical sensitivity on the part of the fish.

The present paper examines the implications of the theory on a quantitative basis.

THE SENSITIVITY OF *GYMNARCHUS NILOTICUS* TO SMALL DIRECT CURRENTS

It has been shown (Lissmann, 1958) that *Gymnarchus niloticus* gives a response (in the form of a sudden movement) when a magnet or an electrified insulator is moved outside the tank. Quantitative experiments have been made to measure the threshold of the response, both to a moving magnet and to a moving electrostatic charge.

A small bar magnet held perpendicularly to the wall of the tank was moved by hand in a vertical direction. A single downward sweep produced a response in the fish if the movement was sufficiently rapid and the distance between the fish and the magnet sufficiently small. With the particular magnet used a response could be elicited at a velocity of about 3 m./sec. when the fish was about 50 cm. from the magnet. The same magnet was then mounted at this distance from a deflexion magnetometer; this gave the value of the magnetic field at the fish. The results are evaluated in Appendix I, where it is shown that a potential gradient of $0{\cdot}03\mu$V./cm. is induced in the water.

Next, an electrostatic charge was moved horizontally just in front of the glass of the tank: again the fish responded if the charge and the velocity were sufficiently high and the distance from the face of the tank sufficiently small. For the electrostatic charge a small aluminium cylinder (a 35 mm. film can) was mounted on an insulated handle, and charged from a Wimshurst machine. The voltage of the

machine was stabilized and measured approximately by letting a continuous stream of sparks flow across a ball-ended spark gap of known spacing. With a voltage of about 60 kV. (spark gap of 4 cm.) a response could be elicited from a fish 50 cm. from the tank face if the velocity of the charge was 3 m./sec. In Appendix I it is shown that this is equivalent to a potential gradient of about 0·04μV./cm. in the water near the fish.

DISCUSSION OF THE DIRECT CURRENT SENSITIVITY OF *GYMNARCHUS NILOTICUS*

From the experiments of the previous section it appears that *Gymnarchus* detects potential gradients of the order of 0·03μV./cm. in the surrounding water. This represents a total voltage from head to tail of about 1μV., and a current density in the fish of about $2 \times 10^{-5}\mu$A./cm.^{2}. It was not possible to investigate by these simple techniques the variation of sensitivity with the relative orientation of the fish and the field; the experiments serve merely to establish its order of magnitude.

Table 1 compares the sensitivity of *Gymnarchus* with that of other fish, giving values of current densities at which responses were noted.

TABLE 1

Species	Current density (μA./cm.^{2})	Source
Phoxinus phoxinus (minnow)	10	Scheminzki (1931)
Cyprinus carpio (carp)	60	Adler (1932)
C. auratus (goldfish)	16	Regnart (1931)
Parasilurus asotus (catfish)	8	Abe (1935)
Gasterosteus aculeatus (stickleback)	110	Johnson (personal communication)
Gymnarchus niloticus	2×10^{-5}	Present authors

It is clear that the sensitivity of *Gymnarchus* is of an entirely different order of magnitude to that of the other fish. This has three important consequences:

(1) Mechanisms of object location involving the detection of minute direct currents become theoretically possible.

(2) The mechanism of perception of electric currents in *Gymnarchus* is likely to be more specifically developed than in most other fish.

(3) Experimental artefacts due to spurious electric currents are likely to be troublesome.

(1) and (2) will be discussed later: some of the implications of (3) will now be considered.

Parker & van Heusen (1917) have shown that currents flow between different parts of a metallic rod immersed in water. They attributed the currents to electrolytic cells formed of the base metal and traces of impurities on its surface. They also found that currents of the order of a few microamperes could be produced in this way: they demonstrated that catfish (*Amiurus nebulosus*) responded to these

currents when metallic rods were brought close to them. It appears from other experiments described in the paper that current densities of the order of several μA./cm.^{2} are needed to evoke a response from the catfish. This agrees with the findings of Abe (1935). *Gymnarchus*, with a sensitivity of at least 10,000 times this value, is likely to be even more responsive to currents generated in this manner.

It is suggested, therefore, that experiments with *Gymnarchus* using metallic electrodes or isolated metallic bodies in contact with the water may well be invalidated by the response of the fish to the small direct currents caused by surface impurities. Thus the response of *Gymnarchus* to the external closing of a circuit between two immersed electrodes (Lissmann, 1951, 1958), the 'trapping' of a fish by an arrangement of copper wires, and the operation of a metallic strip or wire as a punishment device (Lissmann, 1958) can be explained in this way. If currents of the same order of magnitude as those observed by Parker & van Heusen (1917) were set up by these metallic bodies, the current density over nearly the whole aquarium would be greater than the critical value for a response by *Gymnarchus*. The effects would, of course, be maximal close to the metal. Grundfest (1957) reports that gymnotids reacted to metallic conductors near their skin: this is probably a manifestation of the same effect.

It is impossible to be certain that metallic objects do not influence *Gymnarchus* by locally 'short-circuiting' the electric field set up by its own emissions, but this effect is likely to be much smaller than the one described above.

It seems, then, that critical conclusions should only be drawn from experiments in which there are no metallic objects in contact with the water of the aquarium.

RESPONSE TO A STATIONARY MAGNET

Gymnotus carapo can be trained to feed when a stationary permanent magnet is mounted just outside its aquarium and not to feed when the magnet is removed (Lissmann, 1958). A fish swimming through the magnetic field will have currents induced in it in exactly the same manner as currents are induced in a stationary fish by moving a magnet. With the magnet used the field 20 cm. away was about 10 oersted, so that a fish moving at 10 cm./sec. could generate current densities as high as 10^{-4} μA./cm.^{2}. While no definite figures about the sensitivity of *G. carapo* are available, it seems likely from the results on *Gymnarchus niloticus* that currents of this magnitude could readily be perceived, and could be used as the basis for a training experiment.

THE LOCATION OF OBJECTS BY ELECTRIC FISH

The remarkable ability of *Gymnarchus* to avoid obstacles has been noted and attributed to the perception of disturbances to its electric field (Lissmann, 1951). Now that its extremely high sensitivity to direct currents has been shown, it is necessary to re-examine certain apparently unlikely mechanisms for locating objects by means of direct currents. For completeness one non-electric mechanism will also be considered.

(1) Dijkgraaf (1934, 1947) has indicated how objects can be located by most fish by the use of the pressure-detecting function of the lateral line organs. The distribution of water pressure over the surface of a swimming fish depends on the velocity of the fish relative to the water and upon its shape. A nearby obstacle will change the flow pattern of water round the fish, and consequently change the pressure distribution on its surface. On this theory a fish should be incapable of detecting the difference between rigid objects which are geometrically identical, even when made of different material.

(2) The movement of water near a swimming fish will generate small potentials in the earth's magnetic field. Attention was first drawn to this by Thornton (1931), who suggested it as a mechanism by which a fish could locate moving objects such as other fish. He did not perceive, however, that the mechanism could be extended to the detection of stationary objects. For every flow pattern of water there will be a corresponding potential distribution around the fish, the water streamlines coinciding with the electrical equipotentials (Appendix II). A change in the water flow-pattern caused by a stationary object will change the potential distribution around the fish: this effect could be detected and used to locate the object. It can be shown (Appendix II) that the conductivity of the object does not affect the potential distribution, and hence this mechanism again cannot differentiate between geometrically identical objects.

(3) Electric fish emit impulses which set up a flow-pattern of electric current in the surrounding water. Any object with a conductivity different from that of the water will distort this pattern, and hence change the potential distribution around the fish. This change can be used to locate the object. With this mechanism it will be possible to distinguish between geometrically identical objects with differing electrical conductivities. Conversely, it will not be possible to distinguish between dissimilar objects which modify the current distribution in a similar way.

By a series of experiments described later, it has been shown that *Gymnarchus* can distinguish between geometrically identical objects if they have different electrical conductivities, and cannot distinguish between objects which, although geometrically identical and with similar electrical effects, have different internal arrangements.

It is thus clear that in this fish mechanism (3) must play a significant part in the location of objects. The other two cannot be ruled out, but seem unlikely on the following grounds.

(*a*) The differential sensitivity required of the receptors (i.e. least detectable change of pressure ÷ ambient pressure for mechanism (1), and least detectable change of potential ÷ ambient potential for (2) and (3)) is of the same order for the three mechanisms. (3) operates while the fish is stationary, being unaffected by velocity, while (1) and (2) require the fish to be moving. Since in *Gymnarchus* mechanisms (1) and (2) offer no obvious advantages over (3), it does not seem likely that they play any major role in the location of objects.

(*b*) Mechanism (2) depends on the orientation of the fish to the earth's magnetic field. In the equatorial regions where these electric fish originate, the field is

predominantly horizontal, so that large differences might be expected in the potentials developed depending on whether the fish was swimming north to south or east to west.

THE LOCATION OF OBJECTS BY DISTORTION OF THE FISH'S OWN ELECTRIC FIELD

The conclusions of the previous section, taken together with the experimental results given later, make it clear that *Gymnarchus* can detect objects by the distortions they cause in the potential distribution which the fish itself sets up in the water.

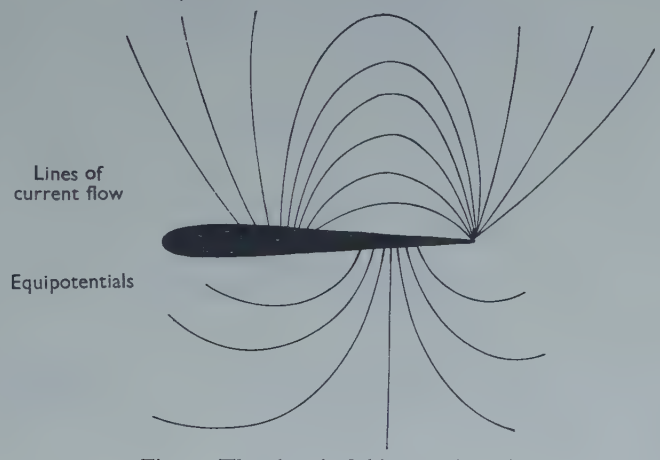


Fig. 1. The electric field around a fish.

For a stationary fish in an infinitely large tank the lines of current flow and the equipotentials are similar to Fig. 1, and correspond approximately to those of a dipole source of current. When an object of a conductivity lower than that of the surrounding water is brought near, the pattern becomes like Fig. 2*a*, while for an object of higher conductivity Fig. 2*b* applies. The distribution of potential around the fish is altered, and therefore the pattern of stimuli received by any electrical receptors on the surface of the fish will be modified. The suggestion that mormyromast-type structures are electrical receptors has been put forward by Lissmann (1958); these structures are situated in the skin and communicate with the surface by jelly-filled canals. The arguments presented here are based on the assumption that these structures are in fact electrical receptors.

In an attempt to find how the potential distribution around a fish would change in the presence of perturbing objects, a model experiment was set up (see Appendix V). A voltage was applied to an electrolytic tank at two points to simulate the dipole field of a fish. The potential in the tank was sampled by pick-up electrodes arranged around these points in the shape of the body of the fish. With delicate and protracted measurements it was possible to locate a large insulating object a few centimetres away from the probe electrodes. However, with any

apparatus of only moderate complexity it was impossible to detect changes in the potential distribution for small and remote objects which could easily be detected by *Gymnarchus*. Some results are, however, quoted later.

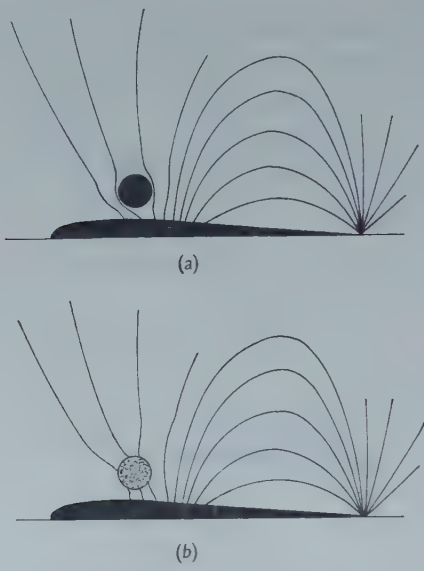


Fig. 2. The electric field in the presence of an object (*a*) of low conductivity, (*b*) of high conductivity.

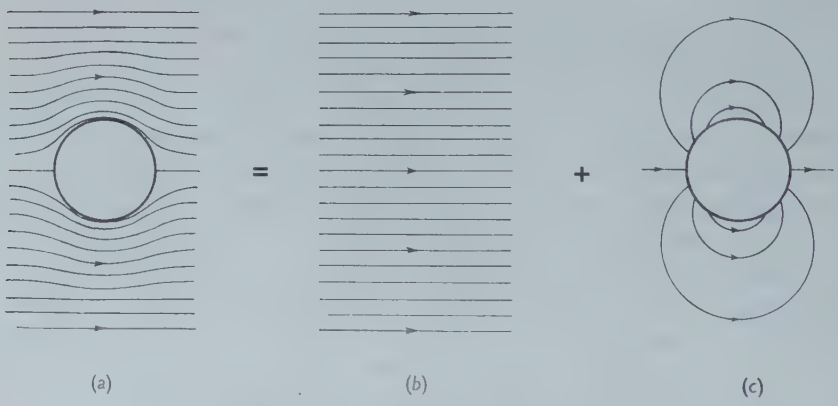


Fig. 3. The resolution of: (*a*) the field in the presence of an object into (*b*) the original field and (*c*) a perturbing field.

A theoretical approach to the problem was next undertaken along the following lines. The field round the fish in the presence of an object can be split up into the original field in the absence of the object, and a perturbing field. This process is illustrated in Fig. 3. The potential at a given point on the body of the fish in the absence of the object is due to the original field; the *change of potential* caused by the object is equal to the *potential* due to the perturbing field. If the

perturbing field can be calculated the pattern of *potential changes* around the body can be derived and hence the sensitivity of the electrical receptors can be assessed.

For objects of certain shapes in a dipole field the perturbing field can readily be calculated since this field is the same as that of an 'image' dipole within the object (Maxwell, 1873). The calculation is carried out in Appendix III for a cylindrical object in a two-dimensional dipole field; this approximates to the conditions obtaining in the experiments described later. The results of the calculation show that the image in the cylinder is a current dipole of moment M' given by

$$\frac{M'}{M} = \left\{a^2 \frac{\sigma_0 - \sigma}{\sigma_0 + \sigma}\right\} \left\{\frac{1}{r_1 r_2}\right\},$$

where a is the radius of the cylinder, σ its electrical conductivity, σ_0 the conductivity of the water, M the moment of the current dipole due to the fish, r_1, r_2 are the distances from the centre of the cylinder to the poles of the original dipole.

The term $\left\{a^2 \frac{\sigma_0 - \sigma}{\sigma_0 + \sigma}\right\}$ is a characteristic of the object only: it will be termed the 'imprimence'* of the cylinder. It is positive if $\sigma < \sigma_0$ and negative if $\sigma > \sigma_0$. For a perfectly insulating cylinder, the imprimence is equal to a^2, while for a perfect conductor it equals $-a^2$.

The second term of the equation for M', i.e. $(1/r_1 r_2)$, depends on the position of the object relative to the fish. For any relative position of the fish (idealized to a dipole) and the cylinder the moment and inclination of the image dipole can be calculated. Assuming that the fish has approximately the same conductivity as the water and that it does not appreciably distort the perturbing field (i.e. does not produce an image of the image), the potential distribution around the fish due to the perturbing field can be calculated.

The effect of the perturbing field on the receptors depends upon the resistance of the jelly-filled canals of the mormyromasts relative to the resistance of the tissue between their proximal ends. It is shown in Appendix IV that when the canal resistance is very high the receptors in effect measure potential. When the canal resistance is very low, the receptors will measure the second derivative of the potential (i.e. the rate of change of potential gradient) around the body of the fish.

The perturbing field due to an object in various positions near a fish has been computed. Before describing the results the assumptions on which the calculation is based will be re-stated: (1) the problem may be treated two-dimensionally; (2) the fish is equivalent to a current dipole; (3) the conductivity of the fish is equal to that of the water. These approximations are not entirely valid for the actual fish; however, the calculations will serve to illustrate qualitatively the effect of objects on the receptor system. In addition, at least the order of magnitude of the perturbation will be indicated.

* In the absence of a suitable word to describe quantitatively the effect of an object on an electric field, the word 'imprimence' has been coined. It is derived from 'impriment' ('something that impresses or imprints') with an ending denoting quantitative measure (cf. 'capacitance'). The use of this coined word avoids the subjective implications of such words as 'electrical perceptibility' or 'visibility'.

The computations have been carried out for a 'fish' of 50 cm. length, and of the general shape of *Gymnarchus*. For simplicity, however, the nose has been drawn elliptical. An insulated cylinder 5 cm. in diameter (i.e. with an imprimence of 6·25 cm.^{2}) is taken as the object; for other values of imprimence the results may be scaled linearly. Results are given for five positions, designated *A–E* in Fig. 4.

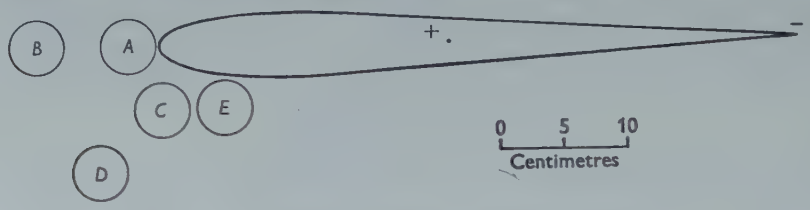


Fig. 4. The position of objects around the model fish.

The strength of the current dipole of the fish has been chosen so that the potentials correspond approximately with those observed in the actual fish (Lissmann, 1951).

Two sets of results are given: (1) for the potential, and (2) for the second derivative of the potential around the surface of the fish. These correspond to the two extremes of relative resistance in the receptor system.

In Fig. 5 the *change of potential* around the fish due to the presence of the object is shown for five positions of the latter. For comparison the *potential* around the fish in the absence of the object is shown to the same scale in Fig. 6.

Fig. 7 shows the *change of second derivative of potential* due to the object. The *second derivative of potential* in the absence of the object is shown in Fig. 8. The large central peak in Fig. 8 is to a great extent artificial and due to the small radius of curvature at the elliptical nose. The peak will be less marked at the nose of an actual fish, since this is flatter than a true ellipse.

The curve *W* shows the *change of potential* (Fig. 5) and the *change of the second derivative of potential* (Fig. 7) which is caused by a 6 cm. displacement of the tail of the fish to the left.

The results lead to the following conclusions:

(*a*) A characteristic disturbance occurs in both the potential and its second derivative around the point on the surface of the fish nearest to the object.

(*b*) The changes of potential are slowly varying and extend over almost the whole of the surface of the fish, while the changes of second derivative are much sharper and more closely confined to the part of the fish nearest to the object.

(*c*) In the second derivative mode the greatest sensitivity is obtained in the 'head-on' position.

(*d*) In the 'head-on' position the width of the peak of second derivative gives information about range: at short ranges a sharp peak flanked by troughs is produced, while at larger ranges the peak is broader. No such discrimination is available in the potential mode.

(*e*) For the second derivative mode the *changes* in stimulation of the receptors due to the presence of the object are of the same order of magnitude as the *total*

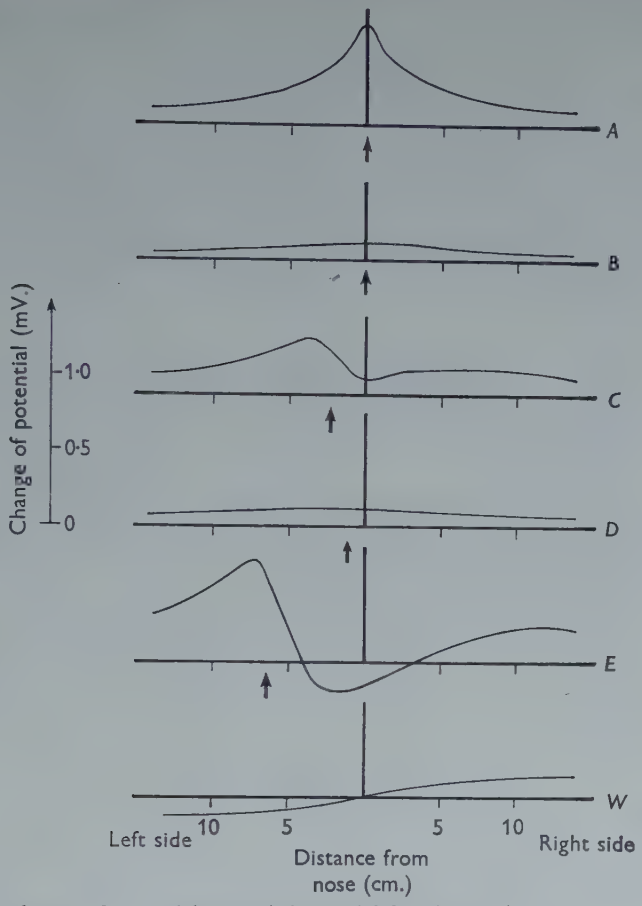


Fig. 5. The change of potential around the model fish due to the presence of an object.

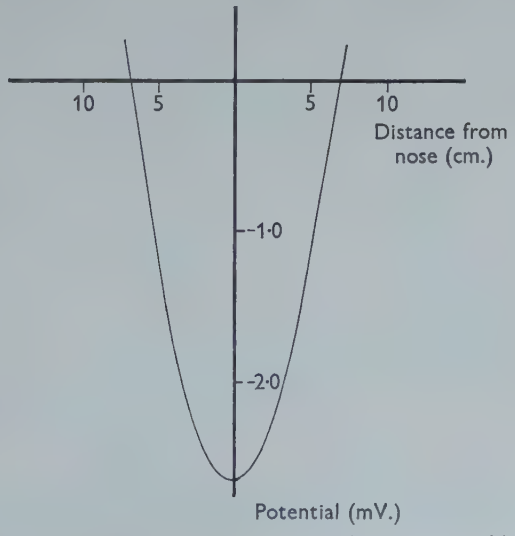


Fig. 6. The potential around the model fish in the absence of an object.

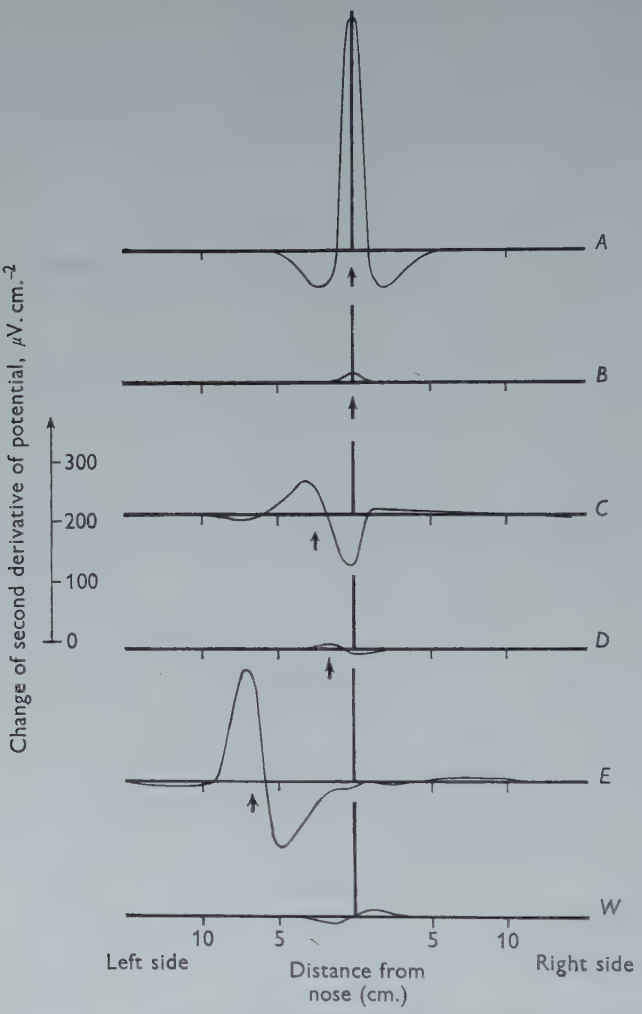


Fig. 7. The change of second derivative of potential around the model fish in the presence of an object.

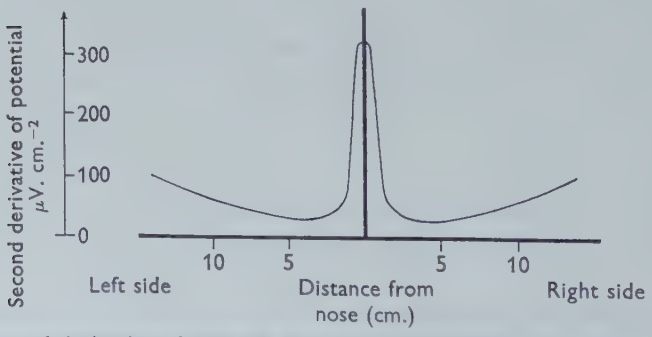


Fig. 8. The second derivative of potential around the model fish in the absence of an object.

stimulation they receive in the absence of the object. For the potential mode the changes are about one-fifth of the total stimulation.

(*f*) Movement of the tail of the fish produces relatively smaller changes of stimulation for the second derivative mode than for the potential mode.

It is clear that the second-derivative mode is capable of the greater location accuracy, and is less disturbed by tail movements. Furthermore, relatively greater changes of stimulation of the receptors are produced by operation in the second-derivative mode. The sensitivity required of the receptors is considered in more detail in a later Section.

EXPERIMENTAL PROGRAMME

The experimental work consisted of a series of training experiments using *Gymnarchus*, and was designed to test the validity of the theory given earlier.

Four sets of experiments were made:

(1) To test whether the fish could distinguish between objects which were geometrically and optically similar but of different electrical conductivity.

(2) To test whether the fish could distinguish between externally similar objects of similar electrical conductivity but of different chemical composition.

(3) To test whether the fish could distinguish between objects of similar 'imprimence' but of different internal construction.

(4) To determine the object of minimum imprimence which could be detected by the fish.

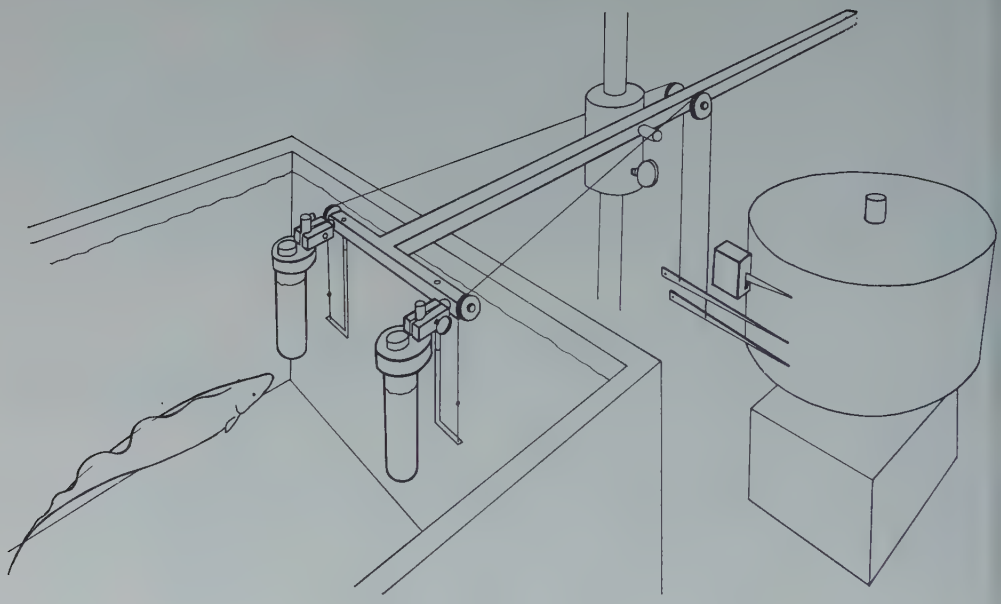
MATERIAL AND METHOD

The training experiments were performed with two specimens of *Gymnarchus*, 52 and 54 cm. in length. Most of the tests were carried out on one of these fish, and the crucial observations checked on the other. The experimental tank measured $120 \times 75 \times 45$ cm., and the water in it was kept at a temperature between 25° and 28° C. The experiments were conducted under ordinary laboratory conditions.

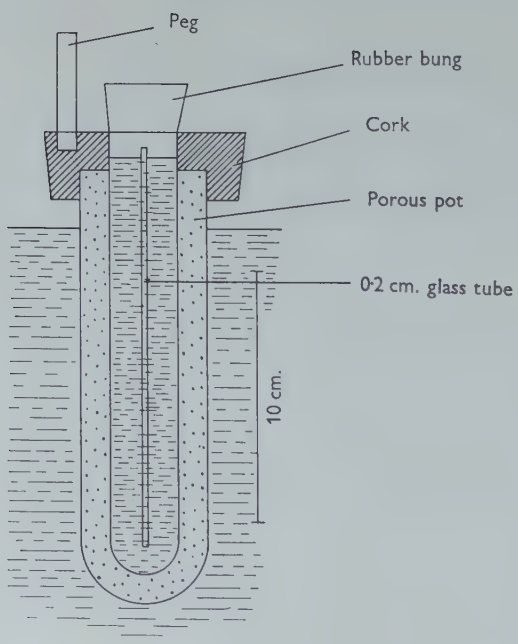
The objects to which the fish were trained were bacteriological filter candles (Berkefeld Filters). These are cylindrical porous pots 15 cm. long and of an outside diameter of 5 cm. The walls, which enclose the lower end, are about 1 cm. thick; the inner cavity has a capacity of about 80 cm.^{3}. The porcelain top of the filter candle was cut off and replaced with a large cork to which the filter was fixed by means of 'Araldite'. This cork had a central hole of the same dimension as the diameter of the cavity in the pot; it could be closed by a rubber bung. The rim of the cork was provided with a peg which could be clamped into a wooden fork on a lever arm, so that two such pots could be dipped simultaneously into the aquarium (Fig. 9). This was always done when the fish was at the far end of the aquarium and facing away from the training site.

This type of object was selected because:

(i) It was found that when such porous pots were placed in water and all the air evacuated from the pores by means of a filter pump, their imprimence was very



(*a*)



(*b*)

Fig. 9. (*a*) Experimental set-up for conditioning experiments.
(*b*) Section through porous pot.

low: notably the very porous 'Nordtmayer' Filters caused very little distortion of a uniform field in an electrolytic tank.

(ii) The presence of a cavity in the pot allowed the inclusion of objects and solutions of varying electrical conductivity and chemical composition.

(iii) The pots were sufficiently strong to withstand the vicious attacks by the fish to which they were exposed during the training experiments.

The training method was of the reward-punishment type. The reward consisted of small pieces of fish, usually one-sixth of a medium-sized minnow. This food was dipped into the tank simultaneously with the porous pot and behind it. It was sewn through with a thread which extended from a Perspex rod fixed to the lever arm holding the pot to a small writing lever writing on a smoked drum (Fig. 9). When the fish accepted the food the pull on the thread recorded on the smoked drum the instant of acceptance. The records also show the instant of presentation and withdrawal of the pots (Fig. 10). Occasionally anomalous records were obtained; this was due mainly to two causes: (i) on some rare occasions the fish bit through the thread holding the food, in which case the tension on the writing lever collapsed suddenly and the subsequent withdrawal of the pot was not recorded; (ii) during presentation of a negative stimulus the more violent attacks by the fish, which were directed against the pot, shook the apparatus to such an extent that acceptance of food was simulated on the record; in actual fact the food was ignored by the fish on these occasions. The automatic recording of the reactions of the fish allowed the experimenter to withdraw in critical tests, thereby reducing the chance of subjective assessment or unconscious signalling.

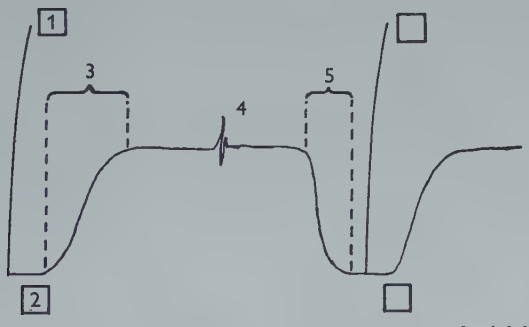


Fig. 10. Diagram of a record in the training experiments. 1, no. of trial in experimental series; 2, type of stimulus; 3, immersion of porous pot and food; 4, acceptance of food; 5, withdrawal of porous pot.

Punishment consisted in chasing away the fish when it approached the food: this was done by immersing a wide wire fork into the water above the fish (see p. 453); in the later stages of this work this punishment was not always sufficient and on occasions the fish had to be knocked gently on the snout with one end of this wire fork.

In some trials the fish had to choose between two porous pots and two sources of food dipped simultaneously into the tank; in another set of experiments the

choice had to be made successively in response to the same pot the contents of which could be altered at will. In the latter case each trial was terminated on presentation of a negative stimulus either after punishment following an attempt to secure the food (noted as incorrect response), or after the fish did not attempt to take the food within a previously determined period of time, which was never less than 30 sec. This was noted as a correct response. Conversely, on presentation of a positive stimulus the trial was ended either as soon as the food had been taken (correct response), or when the fish had refused to accept it within the same period of time (incorrect response).

The order of presentation of the various conditioning stimuli and their position (right or left) was determined by the toss of a coin. Care was exercised to eliminate temperature differences which might be associated with the conditioning stimuli.

QUALITATIVE EXPERIMENTS

In a preliminary series of experiments the fish was trained to distinguish between a porous pot F_1 filled with aquarium water, which represented the positive stimulus, and another pot F_2, saturated and filled with paraffin wax, representing the negative stimulus. Both were offered simultaneously with the food reward behind each. The position of the two pots was varied at random between the right and left corners of the aquarium.

TABLE 2

Trial no.	Position of F_1W	Reaction	Time taken (sec.)	
1	R.	+	12	
2	L.	+	8	
3	L.	+	8	
4	R.	+	10	
5	R.	+	8	
6	L.	+	10	
7	R.	+	10	3 hr. break
8	R.	+	8	
9	R.	+	5	
10	L.	+	10	
11	R.	+	12	
12	L.	+	12	
13	L.	+	18	
14	L.	+	10	

F_1W = positive stimulus (porous pot (F_1) filled with aquarium water).
F_2P = negative stimulus (porous pot (F_2) filled with paraffin wax).

After an exploratory period, during which the training technique was developed, the fish clearly distinguished between the two pots, and towards the end of the training period accepted the food near the pot F_1, usually within 5 to 12 sec. of presentation. An example of one day's trials is given in Table 2.

The variation in the reaction time can be attributed to various causes, but it seems to be partly due to the fact that the fish equally often approached the pot F_2

(negative stimulus) first, before retreating and turning to the other corner where the food was taken behind F_1. It is difficult to state with any degree of accuracy the distance at which the negative stimulus appeared to be recognized, but after a preceding trial which involved punishment the approach was more careful and the point of decision seemed to lie about 20–30 cm. from F_2. With successive trials without punishment the range became gradually closer, but F_2 was rarely approached closer than 5–10 cm., whereas F_1 was frequently touched with the snout both before and after the food had been taken.

Although the two porous pots used in these experiments were superficially similar, they were clearly not identical, and the conclusion that discrimination was accomplished on the basis of the electrical conductivity of the contents cannot be accepted as decisive, unless other senses, notably optical and chemical, can be ruled out. However, when F_1 was replaced by another similar pot F_3, also filled with aquarium water, the fish (without further training) accepted the food behind it without hesitation in five successive trials within an experimental series. Therefore it appears that no optical clues of F_1 were involved in the positive reactions. Moreover, the aquarium water in either of these pots could be replaced with tap water without altering the reactions, and it can be assumed that chemical stimuli from the aquarium water in the pots played no role in these experiments.

TABLE 3

Trial no.	Position of F_1	Contents of F_1	Reaction	Duration of trial (sec.)
1	R.	*W*	+	15
2	L.	*W*	+	18
3	R.	*W*	+	12
4	R.	*A*	−	180
5	R.	*W*	+	12
6	L.	*W*	+	8
7	L.	*A*	−	180
8	L.	*W*	+	20

W=aquarium water; *A*=air; R. and L.=right and left positions of F_1.

That the water-filled pots do not merely represent neutral signals which are differentiated from the negative signals of the wax-filled pot F_2 but represent positive signals, could be demonstrated by emptying the water from F_1 and replacing it with air. This air-filled pot F_1 was now presented simultaneously with the wax-filled pot F_2. Such trials were interspersed with others in which F_1 remained filled with aquarium water. As can be seen from Table 3 the air-filled pot F_1 now represents a negative stimulus, and although the fish repeatedly approached both pots in these experiments, it always retreated and did not attempt to secure the food within the 3 min. that these trials lasted.

Although this negative reaction towards an air-filled porous pot, which had never been associated with punishment, strongly suggests that no chemical associations are involved, it may perhaps be contended that a novel feature in the aquarium—possibly with strange acoustic properties—could be held responsible for the negative behaviour of the fish. To rule out such objections and to exclude any subtle optical and geometrical differences between two pots, it was decided to use only one single pot, and to present it *successively* offering different conditioning stimuli inside it.

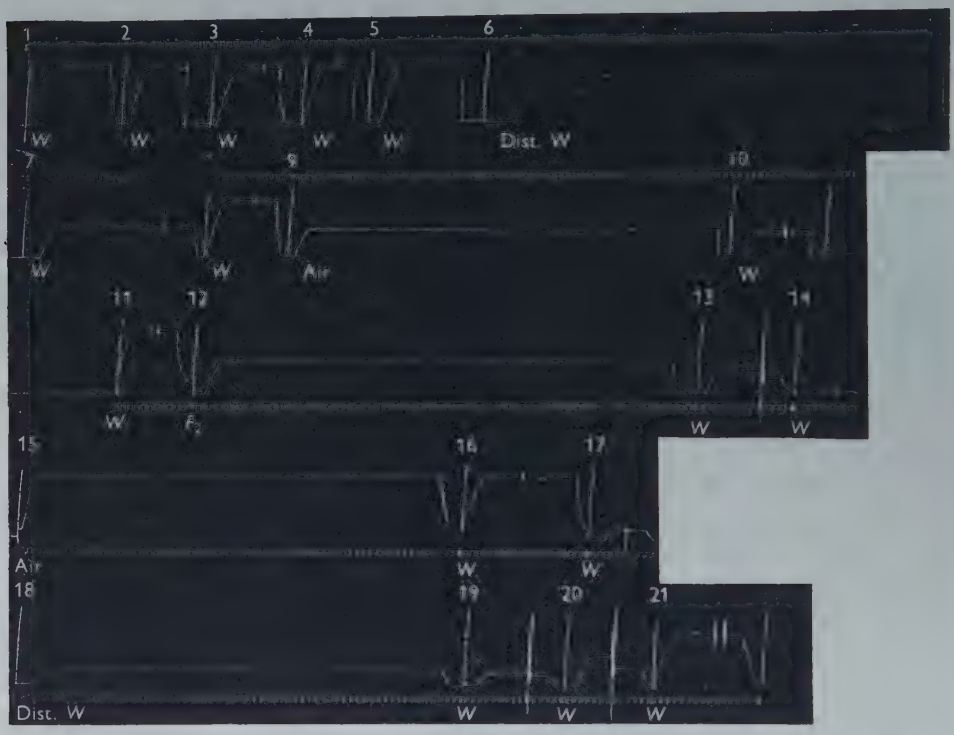


Fig. 11. Record of trials with a fish trained to aquarium water (W = positive stimulus) and paraffin wax (F_2 = negative stimulus) in a porous pot. Note that without further training a negative response is also obtained to air and to distilled water in the porous pot. Time marker = 1 sec.

The conclusions of the previous series of experiments, in which the stimuli were presented simultaneously, were fully confirmed in this way, i.e. the food was now taken within a few seconds when pot F_1 was filled with either aquarium water or tap water, and it was refused within the 60 sec. (later reduced to 30 sec.) after the presentation of a negative stimulus. The negative stimulus to which the fish had been originally trained was the wax-filled pot F_2; without additional training, a negative reaction was also obtained with F_1, if it was filled with air or glass-distilled water which had an electrical conductivity of less than 20μmhos/cm. (Fig. 11). The exchange between the distilled water inside the pot and the aquarium water outside

appears to be negligible within the duration of the experiment (30 sec.). Even after the pot had been left in the aquarium for 60 sec. the conductivity of the distilled water inside was found to be 30μmhos/cm., indicating an interchange of 3% of the pot's volume in this time.

However, it is known that taste receptors are capable of discriminating between tap water and distilled water with a high degree of accuracy (e.g. Liljestrand & Zottermann, 1954). To exclude the possibility that the small interchange of water during the experiment might affect the result the following experiment was carried out. The porous pot F_1 was filled with aquarium water; into this was immersed a glass tube of 2·5 cm. diameter which approximately fitted the central cavity. Again, without additional training, presentation of such a pot evoked negative responses, just as did paraffin wax, air or distilled water. In view of these observations, which were confirmed with both specimens of *Gymnarchus*, it seems hard to avoid the conclusion that the negative stimulus to which these fish have been trained can be attributed to the insulating properties of the contents of the porous pot.

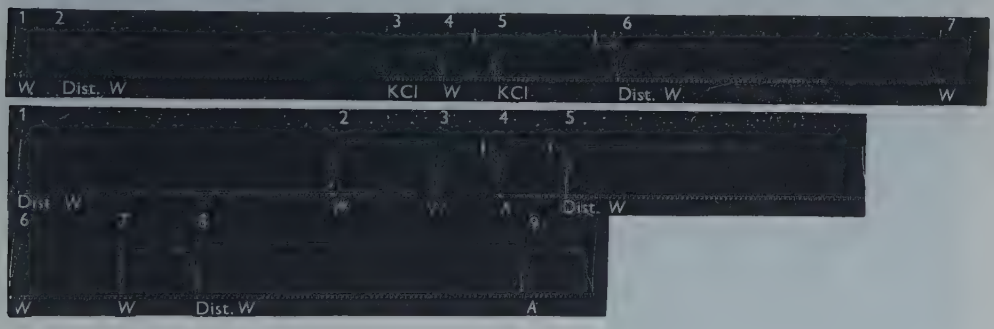


Fig. 12. Record of trials with a fish trained to aquarium water (W = positive stimulus) and distilled water (dist. $W.$ = negative stimulus). Note that, without further training, the positive reaction is also given to KCl and acetic acid (A) solutions if their electrical conductivities approximate to that of the aquarium water. Time marker = 1 sec.

The converse experiment, namely the demonstration that the electrical conductivity and not the presence of any particular ions in the aquarium or tap water represents the positive conditioning stimulus, was carried out by adding various pure solutions to distilled water in the porous pot until the conductivity of the contents approximated to that of the aquarium water. Fig. 12 shows that positive reactions were obtained to solutions of either KCl or acetic acid with conductivities approximately equal to that of the aquarium water.

These results seem to suggest:

(1) That *Gymnarchus* can distinguish between objects which are geometrically and optically identical but which are of different electrical conductivity.

(2) That *Gymnarchus* cannot distinguish between externally identical objects of similar electrical conductivity but of different chemical composition.

QUANTITATIVE EXPERIMENTS

It has been shown in the previous section that *Gymnarchus*, trained to the two extremes (i.e. paraffin wax and aquarium water in a porous pot), will behave towards solutions as if they were aquarium water, provided that their electrical conductivity is approximately that of aquarium water. It was noticed, however, that the behaviour of the fish changed when more dilute solutions of KCl were used. Thus, as long as the conductivity was of the order of 1000 μmhos/cm. (conductivity of aquarium water = 980 μmhos/cm.), the behaviour was indistinguishable from that towards aquarium water, but when trials with solutions of 130 and 260 μmhos/cm. were introduced into a series in which aquarium water had to be distinguished from distilled water, the fish hardly ever accepted the food. On the rare occasions when it did so, this happened after much hesitation just before the 60 sec. trial period had elapsed. On the other hand, it was noted that when trials were conducted in

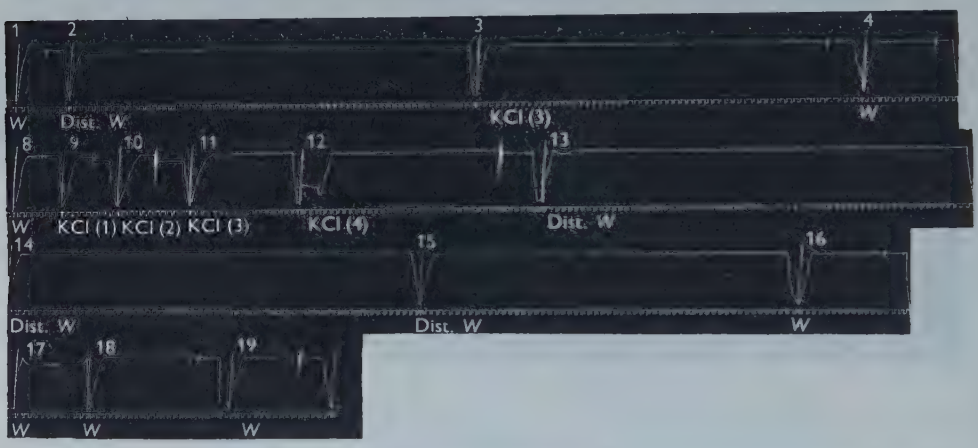


Fig. 13. Record of trials with a fish trained to aquarium water (*W* = positive stimulus) and to distilled water (dist. *W.* = negative stimulus) responding to KCl solutions of various strengths. Electrical conductivity of KCl (1)—1000 μmhos/cm.; KCl (2)—500 μmhos/cm.; KCl (3)—260 μmhos/cm.; KCl (4)—140 μmhos/cm.

rapid succession with gradually decreasing concentrations of KCl solutions the fish was more ready to accept the food even at these lower concentrations. The example in Fig. 13 shows the positive reactions in successive trials towards aquarium water and KCl solutions of a conductivity of 1000, 500, 260 and 140 μmhos/cm., presented in that order. It will be seen from this record that the fish showed progressively more hesitation, and that it refused the food in the three succeeding trials with distilled water, although still hungry and ready to feed, as can be seen from the four following positive responses towards aquarium water.

The regularity in the increase of the reaction time in this record may, in part, be considered as fortuitous, but the increase does suggest that *Gymnarchus*, given sufficient time, is capable of detecting smaller differences in the electrical conductivity of the contents of the porous pot than has become apparent in the quali-

tative experiments described earlier. In order to obtain information about the degree of sensitivity of *Gymnarchus*, a series of training experiments was undertaken in which the fish had to distinguish between mixtures, in different proportions, of aquarium water and distilled water. In Table 4 are summarized the results of training experiments in which the fish (previously trained to distinguish between

TABLE 4

Stimulus	No. of trials	Response		
		Correct	Incorrect	
W	58	52	6	
75 % dist. *W*	39	27	12	
	97	**79**	**18**	$P<0{\cdot}1\,\%$
W	8	8	0	
50 % dist. *W*	7	4	3	
	15	**12**	**3**	$P<0{\cdot}1\,\%$
W	58	54	4	
25 % dist. *W*	50	31	19	
	108	**85**	**23**	$P<0{\cdot}1\,\%$
W	18	12	6	
12·5 % dist. *W*	4	2	2	
	22	**14**	**8**	$P=25\,\%$

Positive stimulus = (*W*) aquarium water.
Negative stimulus = (% dist. *W*) = aquarium water + % distilled water.

aquarium water and distilled water) had to distinguish between aquarium water and aquarium water containing 75, 50, 25 and 12·5% of distilled water included in the pot. In each series training was continued until at least ten successive positive responses were achieved. It is clear from these results that *Gymnarchus* can at least distinguish aquarium water from 75% aquarium water + 25% distilled water. Insufficient data are available in this series for lower dilutions of aquarium water (87·5% W. + 12·5% dist. W.), but the general impression—borne out by later experiments—suggests that even these finer differences can be appreciated.

The conclusion that discrimination was achieved in these experiments by the ability of the fish to sense the electrical conductivity of the solutions and not their chemical composition was supported by a further series of training experiments in which the fish had to differentiate between a porous pot filled with aquarium water and the same pot with aquarium water which also contained centrally placed glass tubes of varying diameters.

A fish, trained to respond negatively to distilled water and positively to aquarium water, also responded negatively, without further training, to aquarium water which contained a glass tube of 2·5 or 2·3 cm. diameter (and occasionally also to tubes of 1·9 and 1·5 cm. diameter), whereas to a tube of 0·9 cm. diameter a positive response was usually given. With further training, however, finer discriminations could be demonstrated. The results of all training experiments to glass tubes of 0·9, 0·6, 0·4, 0·2 and 0·08 cm. are summarized in Table 5.

TABLE 5

Stimulus	No. of trials	Response		
		Correct	Incorrect	
W	34	33	1	
W+0·9 cm.	38	26	16	
	62	**55**	**17**	$P<0{\cdot}1\%$
W	47	43	4	
W+0·6 cm.	49	33	16	
	96	**76**	**20**	$P<0{\cdot}1\%$
W	38	35	3	
W+0·4 cm.	39	22	17	
	77	**57**	**20**	$P<0{\cdot}1\%$
W	167	134	33	
W+0·2 cm.	151	69	82	
	318	**203**	**115**	$P<0{\cdot}1\%$
W	49	35	14	
W+0·08 cm.	47	16	31	
	96	**51**	**45**	$P=60\%$

Positive stimulus = (*W*) aquarium water.
Negative stimulus = (*W* + *x* cm.) aquarium water + glass tubes of varying diameter.

Although it is clear that under such experimental conditions *Gymnarchus* can detect at a distance a glass tube of 0·2 cm. diameter, a closer examination of the records suggests that (i) the performance of the fish is somewhat variable from day to day; (ii) the fish does not remember from one day to the next the more difficult discriminations.

This table, therefore, does not present a completely true picture of the powers of learning and discrimination. In a short test series proportionately more mistakes will be recorded. If two short test series, performed on different days, are added up, the proportion of correct to incorrect reactions would be lower than in a single consecutive series of the same number of trials.

Moreover, in a number of instances the trials were started with the easier task (0·9 cm. tubes) and then made progressively more difficult by reducing the diameter. The learning acquired in the earlier trials was clearly of influence on the later, more difficult, trials.

It is, possible, however, to perform more than 100 trials with a hungry fish in one session. A continuous series of trials with 0·6, 0·35 and 0·2 cm. diameter glass tubes was therefore undertaken in the course of one day during which one may assume that the conditions were more or less constant. The results, summarized in Table 6, show a progressive worsening of the responses with decreasing diameter and they also show that, whereas the responses towards aquarium water remain significantly correct, there is a marked deterioration in the number of correct responses towards the negative stimulus with decreasing diameter of the glass tubes.

Attempts to investigate the ability of the fish to distinguish between two glass tubes of different diameter presented inside the porous pot filled with aquarium water have been undertaken, but have not been pursued to the limits of discrimination.

TABLE 6

Stimulus	No. of trials	Response		
		Correct	Incorrect	
W	7	7	0	
W+0·9 cm.	6	3	3	
	13	**10**	**3**	$P=3\%$
W	8	8	0	
W+0·6 cm.	10	7	3	
	18	**15**	**3**	$P=0{\cdot}1\%$
W	21	19	2	
W+0·4 cm.	19	12	7	
	40	**31**	**9**	$P<0{\cdot}1\%$
W	20	15	5	
W+0·2 cm.	14	6	8	
	34	**21**	**13**	$P=14\%$

Positive stimulus = (*W*) aquarium water.
Negative stimulus = (*W*+*x* cm.) aquarium water + glass tubes of varying diameter.

TABLE 7

Positive stimulus, 0·9 cm. diam. tube	Negative stimulus 1·5 cm. diam. tube
Responses 20 correct; 0 incorrect	Responses 17 correct; 6 incorrect

An example of such an experimental series in which the fish had to distinguish between a glass tube of 0·9 cm. diameter and another of 1·5 cm. are given in Table 7.

If the theory given earlier is correct, the fish should be incapable of distinguishing between a pot containing a glass tube and one containing a water mixture, provided that the mixture is adjusted so that the imprimences are equal in the two cases.

Accordingly, a fish trained to respond positively to a 0·9 cm. glass tube and negatively to a 1·5 cm. tube was presented with various water mixtures introduced into a series of trials with glass tubes. The results are shown in Fig. 14.

It will be seen that both 75 and 50% aquarium water evoke positive reactions, while 40% gives a negative reaction. The corresponding imprimences are shown in Table 8. Thus in terms of imprimence values the boundary between positive and negative reaction lies between 0·2 and 0·6 for glass tubes and between 0·5 and 0·7 for water mixtures. In view of the approximations made in the theory there is little point in attempting to define the boundary more accurately, and the measure of agreement in the results is considered adequate.

TABLE 8

Stimulus	Imprimence, cm.^{2}	Reaction
0·9 cm. tube	0·2	+
1·5 cm. tube	0·6	−
75% aquarium water	0·2	+
50% aquarium water	0·5	+
40% aquarium water	0·7	−

It must be remembered that in all these experiments:

(i) The fish has no means of simultaneous comparison of the two conditioning stimuli and must remember the differences from one trial to the next. Experiments using two externally identical pots with different contents presented simultaneously became inconclusive at fine levels of discrimination, presumably because the imprimences of the pots themselves differed.

(ii) The fish must recognize the differences of the stimuli through the 1 cm. thick walls of the pot, i.e. the imprimence of the pot is added to that of the stimulus in all cases. Furthermore, the conditions under which these experiments were performed were not ideal and various extraneous noises and vibrations clearly influenced the behaviour of the fish.

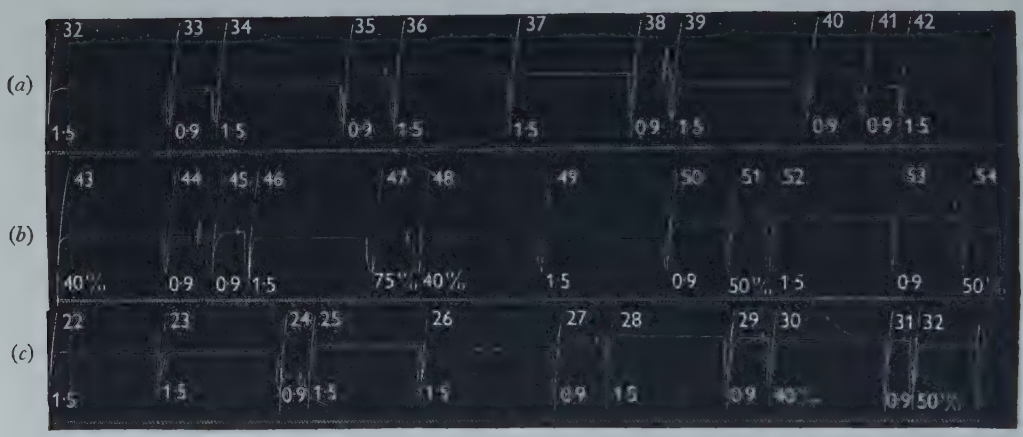


Fig. 14. Record of trials with a fish trained to distinguish a glass tube of 0·9 cm. diameter in a porous pot (0·9 = positive stimulus) from another tube of 1·5 cm. (1·5 = negative stimulus). This fish also responds positively to a mixture of 50% aquarium water + 50% distilled water, and negatively to 40% aquarium water + 60% distilled water. Records (*a*) and (*b*) consecutive series; (*c*) 2 days later. Time marker = 1 sec.

Despite these limitations the general inference from these quantitative experiments is: (1) that *Gymnarchus* cannot distinguish between objects of similar imprimence but of different internal construction; (2) that the object of minimum imprimence which can be detected by the fish is represented by a glass tube about 0·2 cm. in diameter.

Further tests are, however, needed to establish discrimination thresholds for the whole receptive range.

CRITICAL CONSIDERATIONS OF THE METHOD AND GENERAL OBSERVATIONS ON LEARNING AND BEHAVIOUR IN *GYMNARCHUS NILOTICUS*

From time to time in the course of this work the authors had doubts about the validity of the observed results. The delicate reactions of the fish towards external stimuli gave rise to suspicion. For example, the porous pots were clamped to the

lever arm by wooden screws. These screws, when tightened, sometimes produced a squeak in the middle of an experiment. In cases when a weak negative stimulus was presented and the fish hovered near the pot this sound almost invariably led to a sudden attempt to secure the food. After this defect in the apparatus had been eliminated, the same response could be evoked by the slightest touch with the tip of a finger on the slate wall of the aquarium near the pot. The question therefore arose as to whether the responses of the fish were guided or assisted by unconscious signals supplied by the experimenter. This appeared unlikely, because on a few occasions it happened that in a long experimental series with correct responses a single 'mistake' was accounted for by the fact that the experimenter had forgotten to include a glass tube in the pot. This became apparent only after the fish had been mistakenly punished and the bung removed from the pot. After such treatment it was found useful to guide the suspicious fish back to the training site during the next positive stimulus by lightly touching the wall of the aquarium.

In most experiments the fish could have seen only part of the face of the experimenter. Although its indifference to all but the most violent optical stimuli makes it unlikely that it could have received any visual clues, a number of tests were made during which the experimenter was completely out of sight; the reactions of the fish, recorded on the smoked drum, were unaltered. Moreover, violent movements of arm and hand, holding the punishment device above the training site, neither prevented nor delayed the acceptance of food on presentation of a positive stimulus.

Furthermore, in the series of experiments in which *Gymnarchus* had to discriminate between glass tubes of different diameters, trials were undertaken in which the 'experimenter' behind a screen, according to the toss of a coin, included one or the other of the two glass tubes into the porous pot. This was then closed with a rubber bung and passed to the 'handler' who performed the experiment not knowing the diameter, and who then reported back to the 'experimenter' his conclusions by observing the behaviour of the fish. An example of the results of one such test series is given in Table 9. The statistical significance obtained in such tests dispelled all doubts about the validity of the experimental results.

No general conclusions about the learning process in *Gymnarchus* can be drawn from these experiments, since they were performed on only two specimens and involved frequent re-training. It was noted, however, that in the early stages of training there appeared for a number of days a significant number of 'correct' responses during the first six to twelve trials, after which the performance would completely deteriorate. Whether this can be related to the method and number of punishments involved appears uncertain. After punishment the fish usually retreated to the far corner of the tank. Early in the training period the approach in the next trial was undertaken with much caution, often tail first with the tip apparently performing exploratory movements. In the series when different stimuli were presented successively the fish, on presentation of a negative stimulus, first

TABLE 9

No. of trial	Tube diameter known to Experimenter	Tube diameter, reported by Handler
1	1·5	1·5
2	0·9	0·9
3	0·9	0·9
4	1·5	0·9*
5	1·5	1·5
6	0·9	0·9
7	1·5	1·5
8	1·5	1·5
9	0·9	0·9
10	0·9	0·9
11	1·5	1·5
12	0·9	0·9
13	0·9	0·9

* Mistake.

approached the training site and then retreated. Often, however, notably after a number of negative stimuli had been presented in succession, an aggressive mood developed. This usually took the form of a few lateral oscillations, followed by a sudden charge against the pot. Sometimes such attacks were also noted when the usual time of presentation of the stimulus (30 sec.) was extended. An attack also usually took place as soon as the withdrawal of the pot was begun. As can be seen from the records, the withdrawal had to be performed smartly to avoid damage to the pot.

It has been mentioned that *Gymnarchus* does not seem to remember the finer discriminations from one day to the next. On the other hand, evidence is on record that a fish trained to distinguish aquarium water from distilled water in a porous pot showed signs that it remembered this training over a period of four months. When the training was re-started after this interval, the first eight trials gave correct responses; after punishment in the ninth trial very few further mistakes occurred in this series.

THE SECOND DERIVATIVE MODE AS THE MOST PROBABLE MECHANISM; LIMITS OF DETECTION

In a previous section it was shown that a receptor system operating in the second derivative mode gives better discrimination in angle and range than one using the potential mode. Furthermore, from the approximate values of the relevant resistances calculated in Appendix IV, it seems clear that the second derivative mode is the more likely one in *Gymnarchus*. In both modes the receptors have to detect changes in an already existing stimulus; in the typical case in Figs. 5–8 the object changes the existing potential by 15%, while the second derivative of potential changes by 130% for the same object. If the Weber-Fechner Law applies to the receptors, the second derivative mode is clearly capable of higher sensitivity. This mode is also less affected by movements of the tail of the fish.

One point against the second derivative mode is that the actual currents flowing in the receptors are very much smaller than for the potential mode. It is therefore necessary to investigate whether these very small currents are detectable amongst the random fluctuations or 'noise' inherent in every detector system.

The conditioned reflex experiments have demonstrated that *Gymnarchus* can just detect whether a porous pot containing aquarium water also contains a 0·2 cm. diameter glass tube (imprimence of 0·01 cm.^{2}). From Fig. 7, drawn for an imprimence of 6·25 cm.^{2}, it can be calculated that in the most favourable ('head-on') case the fish must be able to detect a change of about 0·6μV.cm.$^{-2}$. The standing value of the second derivative as shown in Fig. 8 is about 300μV.cm.$^{-2}$, so that the relative change (i.e. Weber fraction) is about 0·2%. The thresholds observed in other sense organs are in general higher than 1%, so the detection of this small change would probably present great difficulty. However, as we have noted earlier, the large standing value of second derivative is somewhat artificial and is not likely to be so large in the actual fish. A fairer comparison would be with the object in position *C*; here the change which must be detected is about 0·3μV.cm.$^{-2}$ in a standing value of 25μV.cm.$^{-2}$, i.e. about 1%.

To find the change of current in the receptors, the results of Appendix IV are used, together with the approximate value for the inter-mormyromast resistance calculated there. The change of current is then 0·01$\mu\mu$A. during each pulse, or a mean value (for 1 msec. pulses at 300 c./s.) of 0·003$\mu\mu$A. It is these currents which must be compared with the noise currents in the receptor circuit.

The R.M.S. noise current in a circuit of total resistance R is given by

$$i_{\text{R.M.S.}} = \sqrt{\frac{4KT\Delta f}{R}},$$

where K is Boltzmann's constant, T is the absolute temperature and Δf is the 'bandwidth' of the receptor system, i.e. the frequency range over which the receptors operate. For the resistance in a mormyromast circuit ($\sim$300 kΩ) the noise current $i_{\text{R.M.S.}}$ equals $\frac{1}{4}\sqrt{\Delta f}$ $\mu\mu$A.

The probable bandwidth of the receptor system must be carefully considered. It is usually necessary in problems of this type to consider the law of the detector element—whether linear, square law, etc. In the present case the detector will inevitably be linear for small changes of input, due to the presence of the large standing input. With such a linear system there is no restriction on the bandwidth of the system; it can be made indefinitely small. As the bandwidth is reduced the noise current will decrease, while the current due to the signal will remain the same. The individual pulses will gradually lose their shape, and eventually their identity. Since no information is carried by the pulse nature of the signal, this is of no importance. The reduction of bandwidth to Δf is equivalent to an integration of the signal over a time τ given by $\tau = 1/(2\pi\Delta f)$ and thus the reduction of noise is achieved only at the expense of a long response time.

In addition to temporal integration, the noise can be reduced by spatial integration, i.e. by averaging (possibly in the C.N.S.) the response of many neighbouring

receptors. If n receptors are thus averaged, the noise can be reduced by a factor of $\sqrt{n}$. With both temporal and spatial integration the R.M.S. noise current is given by

$$i_{\text{R.M.S.}} = \frac{1}{4}\sqrt{\frac{1}{2\pi n\tau}}\ \mu\mu\text{A}.$$

There is an optimum value for n depending on the structure of the field pattern due to a typical object. If too many receptors are averaged, the pattern of stimulation of the type shown in Fig. 7 is blurred, with consequent loss of directional accuracy. From that figure it is clear that averaging can be carried out over a strip about 1 cm. wide without much loss of information: in such a strip there may be about 5000 receptors. With this value of n, the noise current becomes

$$i_{\text{R.M.S.}} = \frac{0{\cdot}0015}{\sqrt{\tau}}\ \mu\mu\text{A}.$$

For this current to be smaller than the mean value of the change in current due to the presence of the object (estimated above as $0{\cdot}003\,\mu\mu$A.), τ must be greater than about $\frac{1}{4}$ sec. Such an integration time would not unduly limit the usefulness of the locating mechanism.

Spatial and temporal integration are not unknown in other sense organs (de Vries, 1956), so that their assumption in the present case is plausible. Indeed, some form of temporal integration is essential if the output from the receptors is transmitted along their nerves in the usual way. Without temporal integration it is not possible to transmit information about the amplitude of 1 msec. pulses at 300 c./s. by means of impulses in the sensory nerve where the maximum repetition frequency is of the order of 500 c./s.

Two other mechanisms can be employed to improve the signal-to-noise ratio for the receptor system as a whole. By swimming to and fro near the object the fish may 'scan' the area, giving a field pattern which sweeps over the receptors in a readily identifiable way. This gives an effective increase of integration time, since the information from the receptors may be collected over the time of one whole 'scan'. Furthermore, the small relative change in stimulus would be much more readily detected since several comparisons could be made in a short time.

The other mechanism involves inhibition of the receptors between transmitted pulses. For a 1 msec. pulse with a repetition rate of 300/sec., an improvement of signal-to-noise ratio by a factor of about $\sqrt{3}$ would be obtained in this way. This 'blanking' would have to take place before the point at which temporal integration occurred; there seems no obvious mechanism for carrying this out. Since the signal-to-noise ratio is improved by a relatively small factor, it is unlikely that any very complicated mechanism would be evolved to give 'blanking' of the receptors.

From the point of view of the threshold of object detection the characteristics of the pulses emitted by the fish have little effect. If no 'blanking' takes place only the mean value of the transmitted current affects the threshold. The duration and frequency of the pulses may be dictated by the physiology of the electric organs and possibly by the characteristics of the integration mechanism in the receptors.

COMPARISON OF THE THRESHOLD OF OBJECT DETECTION WITH THE DIRECT CURRENT SENSITIVITY

If the fish is exposed to a uniform electric field, the distribution of potential around it is determined only by the shape of the fish. The second derivative of potential reaches a maximum at the nose: for the model fish of Fig. 4 this is $1{\cdot}3E$ volts cm.$^{-2}$, where E is the uniform potential gradient. Assuming that the same receptors are responsible both for object location and for sensitivity to small direct currents, the threshold for the two effects may be compared.

In an earlier section the threshold for direct currents was established as about $0{\cdot}04\mu$V./cm. The maximum value of the second derivative of potential is thus $0{\cdot}05\mu$V.cm.$^{-2}$. For the detection of objects it was shown that the threshold involved the detection of a change of $0{\cdot}3$–$0{\cdot}6\mu$V.cm.$^{-2}$. In view of the approximate nature of the theory and in view of the experimental accuracy an agreement within an order of magnitude may be considered satisfactory. We may conclude, then, that it is not unreasonable to assume that the same receptors are acting in both cases.

SUMMARY

1. Experiments with moving electrostatic and magnetic fields show that *Gymnarchus niloticus* is sensitive to a potential gradient of about $0{\cdot}03\mu$V./cm.

2. Alternative explanations of some previous experiments are given in terms of this high d.c. sensitivity.

3. An explanation in similar terms is given of experiments in which *Gymnotus carapo* is trained to detect a stationary magnet.

4. The mechanisms available for the location of objects by electric fish are reviewed. It is concluded from the results of a critical experiment (described in a succeeding section) that *Gymnarchus niloticus* can detect objects by the disturbance of its own electric field in the water.

5. The approximate theory of this method of object location is derived. The effect on the receptors of the perturbing field due to an object depends on the electrical properties of the receptors: in the extreme cases the stimulation of the receptors is proportional either to the potential or to its second derivative. Graphs are given showing the effect of an object on the potential and on its second derivative around the surface of the fish.

6. Experiments are described using *Gymnarchus niloticus* which (*a*) confirm that the mechanism of object location employs electric field distortion; (*b*) indicate the limits of the sensitivity of the fish.

7. The second derivative mode appears to be the most probable one operating in *Gymnarchus*. The experimentally determined limits of detection are discussed in relation to the random noise in the receptor circuit: it is concluded that both spatial and temporal integration are likely to be employed.

8. The thresholds for object location and for response to direct currents are compared: it is concluded that the same receptors are probably operating in both cases.

The authors are indebted to Elisabeth Machin for much help with the mathematics and computation.

APPENDIX I. THE CALCULATION OF THE CURRENTS INDUCED BY MOVING ELECTROSTATIC AND MAGNETIC FIELDS

In this Appendix estimates will be made of the current which can be detected by *Gymnarchus niloticus* when moving electrostatic or magnetic fields are generated outside its tank. Since the calculations are intended only to establish the order of magnitude of the effects, the approximations which will be made are not unjustified.

Electrostatic case

It is at first sight not apparent how the movement of an electrostatic charge outside the tank can affect the fish, which is virtually immersed in a perfectly conducting medium. The explanation can best be given in the following terms. When a positive charge is placed in front of the tank, an electric field exists momentarily in the water (i.e. for about $\frac{1}{100}$ μsec.). This causes a current to flow, and negative and positive charges to build up on the front and back faces respectively of the tank. The electric field due to this separation of charge is just equal and opposite in the water to the applied field; therefore no further current flows. The distribution of charge on the water/glass interface at the front of the tank is called the 'induced charge'; it is equal in magnitude and opposite in sign to the charge placed outside the tank.

The explanation of the effect can now be given in terms of this induced charge. Referring to Fig. 15, when the test charge is at A, there is a distribution of induced charge A' with a maximum density opposite A, falling off outwards. If the test charge now moves to B, the induced charge must appear as B'. The movement of induced charge from A' to B' must be through the water, so that currents will flow along such paths as p, q, r.

Hence the calculation involves the following steps:

(a) Describe the distribution of induced charge A'.

(b) Let it move with a velocity equal to that of the test charge.

(c) Calculate the current which thereby flows in the water at the appropriate distance from the front face.

This calculation cannot be solved analytically, but involves numerical integration. This has been done, but it was thought more suitable to present here an approximate calculation which illustrates more clearly the physical principles, and which gives a result only slightly different from that of the more detailed analysis.

The density of induced charge falls off as $1/d^3$ (Fig. 16); 90% of the induced charge is confined within a radius $1{\cdot}8a$ of the point P. Without serious error we may consider all the induced charge confined within a square of side $2a$ centred on P (Fig. 16). The charge density is then $-q/4a^2$. If the test charge moves sideways a distance dx, the charge on $AA'D'D$ disappears, and an equivalent charge appears on $BB'C'C$. A charge dq, given by

$$dq = 2a\,dx\left(-\frac{q}{4a^2}\right) = -\frac{q\,dx}{2a},$$

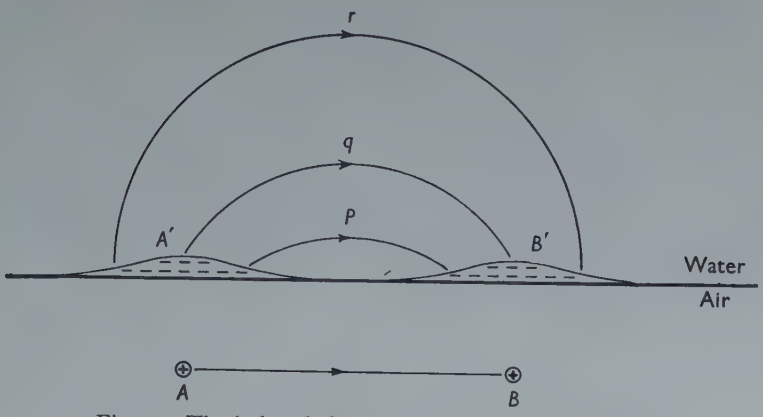


Fig. 15. The induced charge due to a moving test charge.

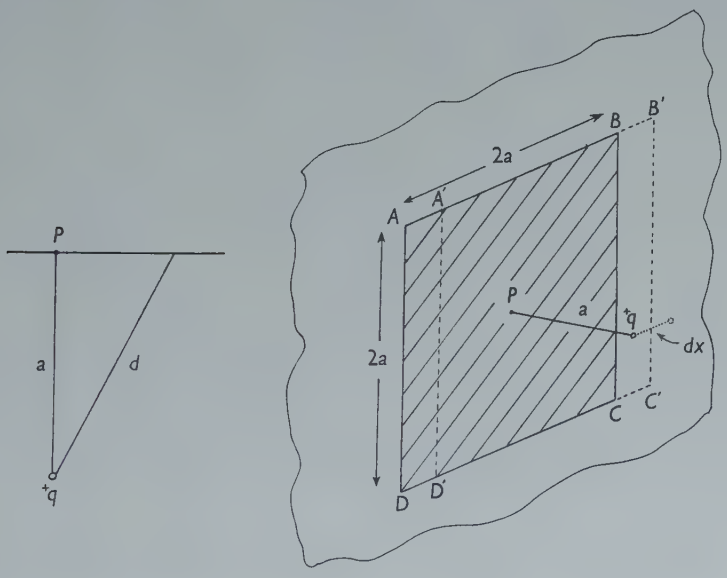


Fig. 16. The geometry of the electrostatic problem.

is thus transferred a distance $2a$. The current flowing from one edge of the square to the other is then given by

$$i=\frac{dq}{dt}=-\frac{q}{2a}\frac{dx}{dt}=-\frac{q}{2a}v,$$

where v is the sideways velocity of the test charge. This flow of current will spread into the tank in the manner shown in Fig. 17, and the current density at a point R within the water can be calculated.

If $d \gg a$, the lines of current flow will correspond to a current dipole of strength M given by

$$M=2ai=\frac{qv}{2a}2a=qv.$$

The current density J in the water at the point R is given by

$$J=\frac{M}{4\pi d^3}=\frac{qv}{4\pi d^3}.$$

In the experiment described in the text, a body of capacity approximately 2 e.s.u. was charged to a voltage of 60 kV. (200 e.s.u.) from a Wimshurst machine. The charge was thus 400 e.s.u. The velocity with which the charge was moved in front of the tank was about 3 m./sec.; a positive reaction could still be obtained from the fish when it was 50 cm. from the tank wall.

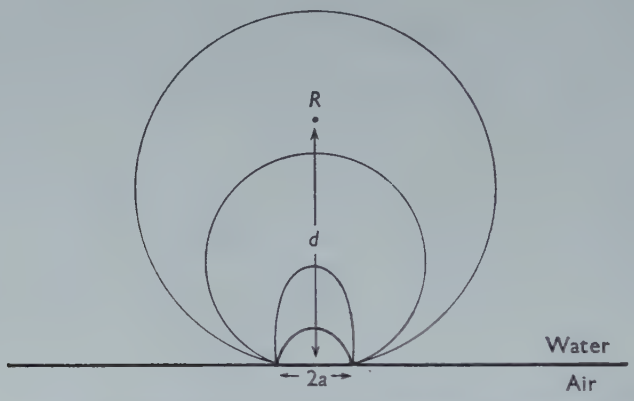


Fig. 17. The spread of current due to the movement of induced charge.

If we assume that the conductivity of the fish is not very different from that of the water, the current density in the fish will be given by the last equation as 0·08 e.s.u., or about $2\times10^{-5}\mu$A./cm.^{2}. If the full calculation is carried out without any of the simplifying assumptions made above, a current density of about half this value, i.e. $10^{-5}\mu$A./cm.^{2} is obtained.

Putting in a value of 500μmhos/cm. for the conductivity of the fish, the potential gradient along it becomes 0·04μV./cm.; for a fish 50 cm. long this represents a total head-to-tail voltage of about 1μV.

Magnetic case

The potential gradient induced in a conductor of unit permeability by a magnetic field H sweeping through it at a velocity v is equal to Hv. In the present experiments, a small bar magnet moved at 3 m./sec. elicited a reaction from a fish 50 cm. away. At this distance, the field was shown by a deflexion magnetometer to be about 0·01 oersted. Hence the potential gradient is equal to 3 e.m.u., or 0·03μV./cm.

The very satisfactory correspondence between this figure and the one (0·04μV./cm.) obtained for the electrostatic case must be regarded as fortuitous, since the approximations made in the calculations will inevitably introduce errors of much greater magnitude.

APPENDIX II. THE POTENTIAL INDUCED IN A FLUID WHICH MOVES IN A MAGNETIC FIELD

The potential gradient, i.e. electric field, induced at any point in a moving fluid is proportional to $vH \sin \theta$, where v is the local velocity of the fluid, H is the magnetic field and θ the angle between them. The direction of the induced electric field is at right angles to both the velocity and magnetic field vectors.

An electric equipotential is a line along which there is no component of electric field; we may therefore identify equipotentials with lines in the fluid *perpendicular* to which there is no component of fluid velocity. Such lines are, of course, the streamlines of the fluid flow. It is clear then that all streamlines must also be electrical equipotentials; the spacing of the lines for equal increments of potential will not necessarily be the same as the spacing of the streamlines.

Since any obstacle to the flow will have one streamline coincident with its boundary, this boundary will also be an equipotential. No current will flow in the obstacle whatever its conductivity; the conductivity of an obstacle therefore cannot affect the potential distribution in the fluid in any way.

APPENDIX III. THE CALCULATION OF THE PERTURBING FIELD BY THE METHOD OF IMAGES

The theory of images in conducting media can best be treated by analogy with electrostatics. It is well known (e.g. Harnwell, 1938) that the equipotentials in a continuous conducting medium are identical with those in free space, and that the lines of current flow coincide with the lines of electrostatic force. The equations of electrostatics can be applied to the conduction case, provided that we substitute for the electrostatic quantities the analogous quantities for conduction. These substitutions are:

Electric induction—$4\pi \times$ current density

Permittivity—Conductivity

The image theory will be developed using electrostatic terminology, and at the end of the calculation the results will be transformed into those appropriate to the conduction case.

The calculation of the perturbing field due to an object of arbitrary shape in a specified field configuration is intractable, so an idealized situation, approximating to the actual one but simpler to analyse, must be used. Since only the order of magnitude of the perturbing field is required the errors introduced by the approximations are not likely to be significant.

In the experiments with *Gymnarchus niloticus* the field of the fish approximated to that of a dipole; the depth of the tank was of the same order as the length of the dipole. Cylindrical objects of about half this length were used. In these circumstances it is a reasonable approximation to treat the problem two-dimensionally. The fish is represented by a line dipole, and the object by an infinite cylinder.

Smythe (1950) gives the image of a line charge in a dielectric cylinder. If a single

charge q is situated at a distance b from the centre of a cylinder of radius a, two images are present:

(*a*) q' at a distance a^2/b from the centre of the cylinder along the radius from q;

(*b*) $-q'$ at the centre of the cylinder.

Here q' is given by

$$q' = q\frac{\epsilon_0 - \epsilon}{\epsilon_0 + \epsilon},$$

where ϵ_0 and ϵ are the permittivities of the surrounding medium and the cylinder respectively. When a dipole is reflected in the cylinder, the two charges at the centre cancel out, leaving the image also as a dipole. This is illustrated in Fig. 18. From the geometry of the figure it follows that the length l' of the image dipole is given by

$$l' = \frac{a^2 l}{r_1 r_1},$$

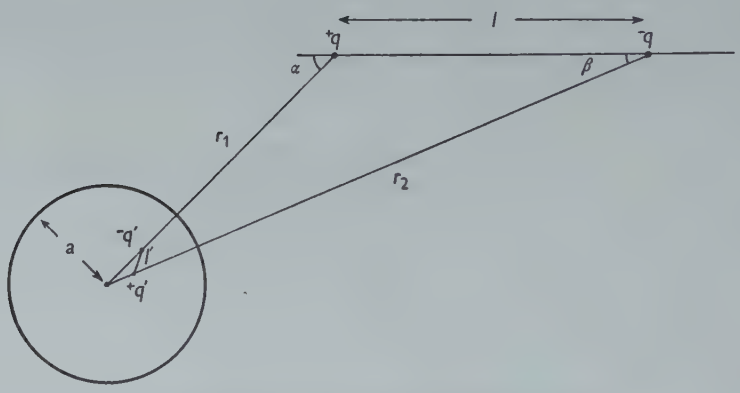


Fig. 18. The image of a dipole in a dielectric cylinder,

and its inclination γ to the original dipole axis by

$$\gamma = \alpha + \beta.$$

The moment M' of the image dipole is thus given by

$$M' = q'l' = q\frac{\epsilon_0 - \epsilon}{\epsilon_0 + \epsilon}\frac{a^2 l}{r_1 r_2} = \frac{M}{r_1 r_2}a^2\frac{\epsilon_0 - \epsilon}{\epsilon_0 + \epsilon},$$

where M is the moment of the original dipole.

Substituting σ, the conductivity, for ϵ, the permittivity, it follows that:

The image of a current dipole of moment M in a cylinder of radius a is itself a dipole of moment M' at an angle γ to the original dipole axis, where

$$M' = \frac{M}{r_1 r_2}a^2\frac{\sigma_0 - \sigma}{\sigma_0 + \sigma} \quad \text{and} \quad \gamma = \alpha + \beta.$$

Here σ_0 = conductivity of surrounding medium; σ = conductivity of cylinder; r_1, r_2 = distances from centre of cylinder to the poles of the original dipole; α, β = angles to the axis of the lines joining the poles of the original dipole and the centre of the cylinder.

APPENDIX IV. THE EFFECT OF MORMYROMAST RESISTANCE

The electric currents flowing into any mormyromast will depend not only on the distribution of potential in the neighbouring water, but also on the relative resistances of the jelly-filled canals and the intervening tissue. The situation may be represented in the one-dimensional case by the equivalent circuit of Fig. 19.

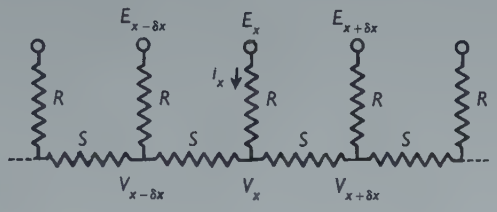


Fig. 19. The equivalent circuit of the receptor system.

Here R is the resistance of the jelly-filled canals and S is the resistance of the tissue between their proximal ends. The terminals are 'sampling probes' at the same potential E as the surrounding water. If V is the potential at the proximal end of R, and i is the current flowing in it, then

$$i=\frac{V_x-V_{x-\delta x}}{S}+\frac{V_x-V_{x+\delta x}}{S}=-\frac{(\delta x)^2}{S}\frac{d^2V}{dx^2}.$$

Furthermore,

$$i=\frac{E-V}{R}.$$

From these equations

$$Si-R\frac{d^2i}{dx^2}(\delta x)^2=-\frac{d^2E}{dx^2}(\delta x)^2.$$

The full solution of this equation must be expressed in terms of Fourier series, but the two extreme cases will be considered.

(*a*) $R=0$. Here

$$i=-\frac{1}{S}\frac{d^2E}{dx^2}(\delta x)^2.$$

(*b*) $S=0$. Here

$$R\frac{d^2i}{dx^2}=\frac{d^2E}{dx^2} \quad \text{or} \quad i=\frac{E-C_1}{R},$$

where C_1 is the mean potential inside the fish.

Thus when the resistance of the jelly-filled canals is low, the current in them (i.e. the stimulus to the receptor at the proximal end) will be proportional to the second derivative of the potential in the neighbourhood. If, on the other hand, the tissue resistance is low, the current (i.e. stimulus) will be proportional to the local potential.

It can be shown that, if

$$\frac{S}{R}\gg\frac{1}{E}\frac{d^2E}{dx^2}(\delta x)^2,$$

the receptor system will operate effectively in the 'second derivative' mode, while if

$$\frac{S}{R} \ll \frac{1}{E}\frac{d^2E}{dx^2}(\delta x)^2$$

it will operate in the 'potential' mode.

In *Staetogenys elegans* the jelly-filled canals were found to be about 0·1 mm. long and 0·02 mm. diameter; if the conductivity of the contents is the same as that of the slime measured by Thornton (1931) they would have a resistance of about 300 kΩ. Assuming a similar value for *Gymnarchus*, the value of S corresponding to operation in the two modes can be calculated. From Figs. 5–8, $\frac{1}{E}\frac{d^2E}{dx^2}$ is of the order of 0·1 cm.$^{-2}$; furthermore, δx, the distance between neighbouring mormyromasts, is of the order of 0·2 mm. From the inequalities given above, the operation will be in either the potential or second derivative mode, depending on whether S, the tissue resistance, is much smaller or much greater than 15 ohms.

A very rough value for S may be obtained by considering the proximal ends of the mormyromasts as spheres of 0·1 mm. diameter embedded in material with a conductivity of 2000μmhos/cm. (the value given by Thornton (1931) for fish skin). This gives a value for S of about 10,000 ohms, indicating that the second derivative mode is the most likely one.

APPENDIX V. MODEL EXPERIMENT USING AN ELECTROLYTIC TANK ANALOGUE

A full-scale electrolytic tank analogue of *Gymnarchus niloticus* was set up in order to measure the changes of potential produced by objects.

Electrodes representing (i) the transmitting dipole (two carbon rods) and (ii) the receptors around the nose (25 silver-tipped wires) were mounted in the appropriate positions on a Perspex sheet. The electrodes were immersed in a shallow tank of tap water.

The equipment could be operated in either the 'potential' or 'second derivative' mode. The circuits for these two modes are shown in Figs. 20*a* and 20*b*. A ganged multi-way switch selected the appropriate electrodes for connexion to the circuit.

A wax-filled pot of the type used for the conditioning experiments with *Gymnarchus* could be mounted in various positions relative to the 'nose'.

Readings either of potential or of its second difference were taken for each position of the switch, both with and without the object present. The difference between the two sets of readings gave the effect due to the object.

The experimental results are shown in Fig. 21. They agree in general shape with the theoretical results of Figs. 5 and 7, but it is clear that the experimental errors are too large for the method to be of much value.

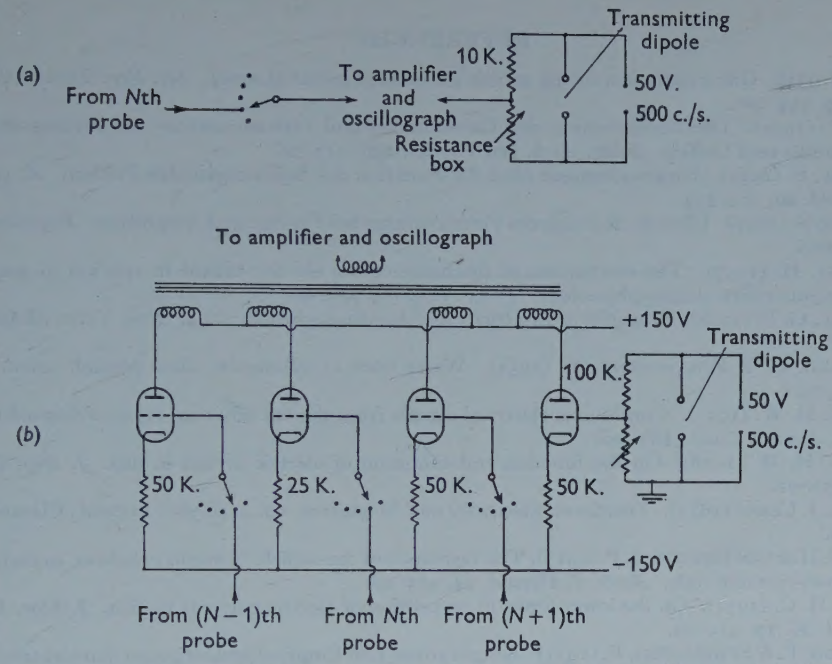


Fig. 20. Circuit of the electrolytic tank analogue: (*a*) potential mode, (*b*) second derivative mode.

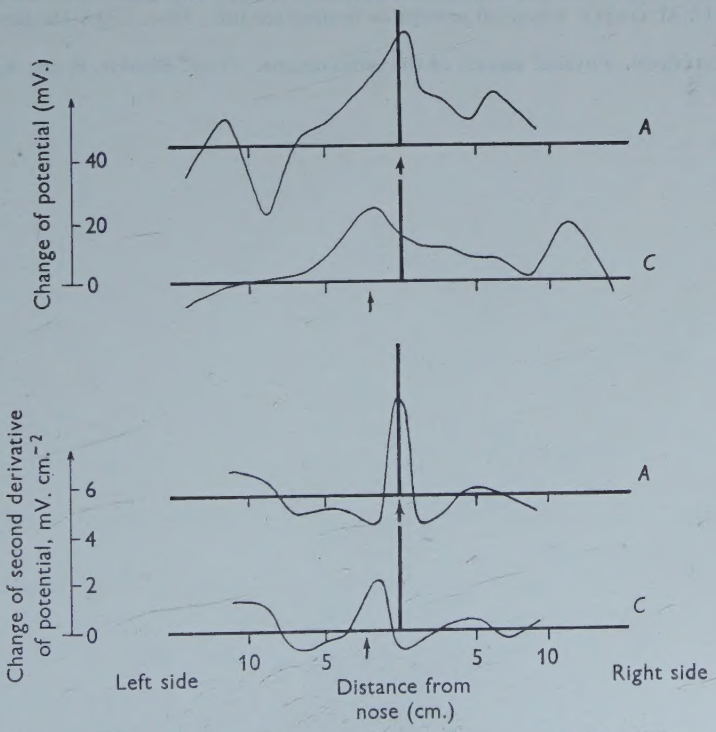


Fig. 21. The change of potential and its second derivative due to the presence of an object. *A* and *C* denote pot positions similar to *A* and *C* of Fig. 4.

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